Biomarkers for the Differential Diagnosis of Pancreatitis and Pancreatic Cancer

The present invention provides biomolecules and the use of these biomolecules for the differential diagnosis of pancreatitis and pancreatic cancer. In specific embodiments, the biomolecules are characterised by mass profiles generated by contacting a test and/or biological sample with an anion exchange surface under specific binding conditions and detecting said biomolecules using gas phase ion spectrometry. The biomolecules used according to the invention are preferably proteins or polypeptides. Furthermore, preferred test and/or biological samples are blood serum samples and are of human origin.

BACKGROUND TO THE INVENTION

Pancreatitis

Pancreatitis is a disease wherein the organ becomes inflamed as a result of cellular injury. There are 2 forms of this disease, acute and chronic pancreatitis, each being clinically and histologically distinct from the other. Morphologically, acute pancreatitis is characterised by the sudden onset of enzymatic necrosis (auto-digestion) and inflammation of pancreatic tissue, and is typically a disease wherein the patient recovers completely. Acute pancreatitis is a relatively common disorder with an incidence rate in Western countries of 10 to 20 cases per 100,000 individuals. In chronic pancreatitis it is the continued loss of pancreatic parenchyma, followed by irregular distribution of fibrosis, that distinguishes it from its acute form. Currently, the incidence of chronic pancreatitis is estimated to be approximately 3-5 cases per 100,000 individuals. In contrast to acute pancreatitis, this form of the disease results from a continuous or relapsing inflammation of the pancreas. Although the typical cause of chronic pancreatitis is substance abuse e.g. alcohol over many years, this form of pancreatitis may also be triggered by a single acute attack, particularly if the pancreatic ducts are damaged. For the most part, chronic pancreatitis is a disabling disease rather than a life-threatening condition and, unlike its acute form, it does not resolve itself. Furthermore, there appears to be an increased risk of pancreatic cancer in patients with chronic pancreatitis.

Pancreatic Cancer

Currently, there is a general agreement that pancreatic cancer arises from alterations in the pancreatic ductal epithelium. However, there is also evidence that trans-differentiation of other pancreatic cell types such as acinar cells, might serve as an alternative route to pancreatic cancer. In the United States approximately 29,000 people are diagnosed with cancer of the pancreas each year; whereas in Europe this total reaches to more than 60,000. Pancreatic adenocarcinoma is the most common type of cancer of the pancreas, accounting for greater than 85% of pancreatic neoplasms. It also accounts for 2-3% of all cancer types, and is the fifth most frequent cause of cancer-related deaths, preceded only by lung, colon, breast and prostate cancers.

Diagnostics

Despite advances in the area of diagnostics, pancreatitis and pancreatic cancer are difficult to diagnose. More so, the distinction between chronic pancreatitis and pancreatic cancer is even more difficult. Despite several available diagnostic tools, there are no non-invasive tests that can confidentially confirm the presence of any of these diseases. For example, serum concentrations of amylase and lipase, the most commonly measured pancreatic enzymes, are not reliable in the diagnosis of acute pancreatitis since they have limitations in their diagnostic sensitivities and specificities. A new urinary test strip that utilises trypsinogen-2 as a marker may play a role in the diagnosis of acute pancreatitis. Based on median sensitivities and specificities, an elevated trypsin level has a better likelihood ratio for detecting pancreatitis than the amylase level, and is probably the most accurate serum indicator for acute pancreatitis. CA19-9, a carbohydrate antigen elevated in cases of chronic pancreatitis, is used as a standard test for detection of the disease. Unfortunately, the specificity of this assay is only around 50%.

Furthermore, the serological diagnosis of pancreatic cancer using various tumour markers such as CEA, CA242 and CA19-9, has been assessed. Unfortunately, the sensitivity and specificity of these markers appear to be insufficient for the differentiation of pancreatic cancer and chronic pancreatitis. At present, there are no commercially available serum markers that can be used to confidently distinguish pancreatic cancer from chronic pancreatitis. In addition, pancreatic cancer is usually diagnosed late in the course of the disease, when the tumour has spread locally or metastasised and curable resection (so far the only available effective therapy) is no longer possible.

Due to a lack of specific symptoms and limitations in diagnostic methods, pancreatic cancer often eludes detection during early stages. Even if pancreatic cancer is suspected, it is difficult to diagnose early stages using conventional methods. As a cure, curable resection is currently only possible if the disease is detected early enough, a simple and reliable serological screening test using disease-specific biomarkers/biomarker patterns would be of tremendous advantage. Such a diagnostic tool would be of great assistance to the clinician, it would aid in the screening and detection of occult disease in asymptomatic persons, which may reduce mortality through early treatment. It would also be helpful in excluding the presence of disease in symptomatic persons and to assist in differential diagnosis. Finally, such a tool could be useful when (1) evaluating the severity of disease, (2) estimating a prognosis, (3) monitoring the course of disease (i.e. progression, stability, or resolution), and (4) detecting disease recurrence.

Currently, many groups are utilising proteomic technologies to comparatively analyse the differences in protein levels in diseased patients as compared to non-diseased subjects, in the hopes of developing diagnostic markers that could assist the practicing clinician in the management of a given disease. Currently, the standard method of proteome analysis has been two dimensional (2D) gel electrophoresis, which is an invaluable tool for the separation and identification of proteins. This method is also an effective tool for the identification of aberrantly expressed proteins in a variety of tissue samples. Unfortunately, the analysis of data generated by 2D-gel electrophoresis is labour-intensive and requires large quantities of material for protein analysis, thereby rendering it impractical for routine clinical use.

Through the introduction of SELDI (surface enhanced laser desorption ionization), a modification of MALDI-TOF (matrix-assisted laser desorption ionization/time of flight) which is a mass spectrometry technique that allows for the simultaneous analysis of multiple proteins in one sample, this tool has been achieved. Small amounts of proteins can be directly bound to a biochip, carrying spots with different types of chromatographic material, including those with hydrophobic, hydrophilic, cation-exchanging and anion-exchanging characteristics. This approach has been proven to be very useful to identify proteins and protein patterns (profiles) in various biological fluids (Ciphergen Inc.).

To date, specific serological biomarkers for the detection of breast and prostate cancers (patents WO0223200, WO03058198 and WO0125791 from Ciphergen, respectively) have been identified using the above mentioned SELDI technology. Unfortunately, due to the nature of sample testing, the biomarkers identified can only be used to diagnose a patient as having a specific cancer (either breast or prostate) versus not having the disease at all. For example, whereas the test samples analysed in WO03058198 (Ciphergen) and WO0223200 (Ciphergen) were taken from patients with late-stage breast cancer (stages III and IV), the control samples were taken from patients with undetectable breast cancer. The biomarkers identified are neither grade-specific nor can they detect the disease at its earliest stages (stage I and II), and thereby would not allow for effective patient-specific diagnosis and/or treatment of the disease. Moreover, serological biomarkers that can differentiate between the presence of chronic pancreatitis and pancreatic cancer have not yet been identified using the SELDI proteomic technology.

Again, there is a critical need to develop a simple, non-invasive, reliable and inexpensive method for the effective detection of pancreatic cancer at its early stages. Preferably, such a diagnostic method should be able to detect early-stage pancreatic cancer, as well as distinguish between the later stages or grades of the disease. Furthermore, such a diagnostic tool should be able to differentiation between chronic pancreatitis and pancreatic cancer. With such valuable information, medical practitioners would be able to tailor patient therapies for optimum treatment of the disease.

The present invention addresses this difficulty with the development of a non-invasive diagnostic tool for the differential diagnosis of pancreatitis and pancreatic cancer.

SUMMARY OF THE INVENTION

The present invention relates to methods for the differential diagnosis of pancreatitis and pancreatic cancer by detecting one or more differentially expressed biomolecules within a test sample of a given subject, comparing results with samples from healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer, or subjects having pancreatitis, wherein the comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer or pancreatitis.

The present invention provides a method for the differential diagnosis of pancreatitis and pancreatic cancer, *in vitro*, comprising obtaining a test sample from a subject, contacting test sample with a biologically active surface under specific binding conditions, allowing for biomolecules present within the test sample to bind to the biologically active surface, detecting one or more bound biomolecules using mass spectrometry thereby generating a mass profile of said test sample, transforming data into a computer-readable form, and comparing said mass profile against a database containing mass profiles specific for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having pancreatic cancer, subjects having metastasised pancreatic cancers, or subjects having pancreatitis, wherein the comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer or pancreatitis.

In one embodiment the invention provides a database comprising of mass profiles of biological samples from healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer, or subjects having pancreatitis.

Within the same embodiment the database is generated by obtaining biological samples from healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer, and subjects having pancreatitis, contacting said biological samples with a biologically active surface under specific binding conditions, allowing the biomolecules within the biological sample to bind to said biologically active surface, detecting one or more bound biomolecules using mass spectrometry thereby generating a mass profile of said biological samples, transforming data into a computer-readable form, and applying a mathematical algorithm to classify the mass profiles as specific for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having pancreatic cancer, subjects having metastasised pancreatic cancer, and subjects having pancreatitis.

In specific embodiments, the present invention provides biomolecules having a molecular mass selected from the group consisting of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, and 28124 Da ± 141 Da. The biomolecules having said molecular masses are detected by contacting a test and/or biological sample with a biologically active surface comprising an adsorbent under specific binding conditions and further analysed by gas phase ion spectrometry. Preferably the adsorbent used is comprised of positively charged quaternary ammonium groups (anion exchange surface).

In specific embodiments, the invention provides specific binding conditions for the detection of biomolecules within a sample. In preferred embodiments, a sample is diluted 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine, and then diluted again 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at a pH 8.5 at 0 to 4°C. The treated sample is then contacted with a biologically active surface comprising of positively charged (cationic) quaternary ammonium groups (anion exchanging), incubated for 120 minutes at 20 to 24°C, and the bound biomolecules are detected using gas phase ion spectrometry.

In an alternative embodiment, the invention provides a method for the differential diagnosis of pancreatitis and/or pancreatic cancer comprising detecting of one or more differentially expressed biomolecules within a sample. This method comprises obtaining a test sample from a subject, contacting said sample with a binding molecule specific for a differentially expressed polypeptide, detecting an interaction between the binding molecule and its specific polypeptide, wherein the detection of an interaction indicates the presence or absence of said polypeptide, thereby allowing for the differential diagnosis of a subject as healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer or pancreatitis. Preferably, binding molecules are antibodies specific for said polypeptides.

The biomolecules related to the invention, having a molecular mass selected from the group consisting of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da,.11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da,, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, and 28124 Da ± 141 Da, and may include, but are not limited to, molecules comprising nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides (DNA or RNA), polypeptides, proteins, antibodies, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins). Preferably said biomolecules are proteins, polypeptides, or fragments thereof.

In yet another embodiment, the invention provides a method for the identification of biomolecules within a sample, provided that the biomolecules are proteins, polypeptides or fragments thereof, comprising: chromatography and fractionation, analysis of fractions for the presence of said differentially expressed proteins and/or fragments thereof, using a biologically active surface, further analysis using mass spectrometry to obtain amino acid sequences encoding said proteins and/or fragments thereof, and searching amino acid sequence databases of known proteins to identify said differentially expressed proteins by amino acid sequence comparison. Preferably the method of chromatography is high performance liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC). Furthermore, the mass spectrometry used is selected from the group of matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), surface enhanced laser desorption ionisation/time of flight (SELDI-TOF), liquid chromatography, MS-MS, or ESI-MS.

Furthermore, the invention provides kits for the differential diagnosis of pancreatitis and /or pancreatic cancer.

The test or biological samples used according to the invention may be of blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract origin. Preferably, the test and/or biological samples are blood serum samples, and are isolated from subjects of mammalian origin, preferably of human origin.

DESCRIPTION OF FIGURES

Figure 1. Comparison of protein mass spectra processed on the anion exchange surface of a SAX2 ProteinChip array comprised of cationic quaternary ammonium groups. Protein mass spectra obtained from sera of control patients (C1 and C2), suffering from chronic inflammation of the pancreas (pancreatitis) and of patients with pancreatic cancer (T1 and T2) are shown. A representative differentially expressed protein (m/z= 9099 Da) is highlighted possessing high importance within the generated classifiers (ensemble of decision trees) according to overall improvement, see Tables 1-4. The X-axis shows the mass/charge (m/z) ratio, which is equivalent to the apparent molecular mass of the corresponding biomolecule. The Y-axis shows the normalized relative signal intensity of the peak in the examined serum samples.

Figure 2A - E. Scatter plots of peak clusters belonging to differentially expressed proteins included in the classifier. Peak clusters are aligned along the vertical axis, e.g. M1624.44 denotes the peak cluster with characteristic mass 1624 Da. The horizontal axis shows the raw relative signal intensity of the peaks in the examined serum samples. Here, "raw" refers the non-logarithmic and not additionally normalized intensities, see Figure 3 and 4 for further intensity

transformations. □ T (Tumour): Pancreatic cancer patients' serum samples. ○ C (Control): Pancreatitis patients' serum samples.

Figure 3A - E. Scatter plots of peak clusters belonging to differentially expressed proteins included in the classifier. Peak clusters are aligned along the vertical axis, e.g. M1624.44 denotes the peak cluster with characteristic mass 1624 Da. The horizontal axis shows the logarithmic normalized relative signal intensity of the peaks in the examined serum samples. For each mass, intensities were first shifted to entirely positive values and then normalized by dividing the intensity values by the average intensity of that mass. Finally, the base 2 logarithm was taken. Accordingly, zero logarithmic normalized relative intensity refers to mean peak cluster intensity, and logarithmic normalized relative intensities of +1 and -1 mean two-fold over- and under-expression relative to mean peak cluster intensity, respectively. □ T (Tumour): Pancreatic cancer patients' serum samples. ○ C (Control): Pancreatitis patients' serum samples.

Figure 4A - E. Additionally scaled scatter plots of peak clusters belonging to differentially expressed proteins included in the classifier. Peak clusters are aligned along the vertical axis, e.g. M1624.44 denotes the peak cluster with characteristic mass 1624 Da. As in Figure 3, the Y-axis shows the logarithmic normalized relative signal intensity of the peaks in the examined serum samples. However, intensities were additionally (shifted and) scaled so that the intensities of each peak cluster cover the entire horizontal range. Thereby, the minimum and maximum intensities of all masses are aligned on the left and right edge of the plot, respectively. This allows to better visualize the extend of class overlap. □ T (Tumour): Pancreatic cancer patients' serum samples. ○ C (Control): Pancreatitis patients' serum samples.

Figure 5. Complexity of proof-of-principle classifier. The histogram visualizes the distribution of the number of decision tree variables (peaks, clusters) for the obtained proof-of-principle classifier for pancreatitis versus pancreatic cancer. 6-8 variables per decision tree are typical.

Figure 6. Variable importance of the proof-of-principle classifier. The histograms visualize how often a variable (mass) is employed in the proof-of-principle classifier. The frequency of variable selection is presented in histogram form for the first 10 hierarchical level (a-j) and for all hierarchical levels taken together (k).

Figure 7. Complexity of final classifier. The histogram visualizes the distribution of the number of decision tree variables (peaks, clusters) for the obtained final classifier. 11-13 variables per decision tree are typical.

Figure 8. Variable importance of final classifier. The histogram visualizes how often a variable (mass) is employed in the final classifier. The frequency of variable selection is presented in histogram form for each of the first 10 hierarchical levels (a-j) and for all hierarchical levels taken together (k).

DESCRIPTION OF THE INVENTION

It is to be understood that the present invention is not limited to the particular materials and methods described or equipment, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

It should be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "an antibody" is a reference to one or more antibodies and derivatives thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art. Although any materials and methods, or equipment comparable to those specifically described herein can be used to practice or test the present invention, the preferred equipment, materials and methods are described below. All publications mentioned herein are cited for the purpose of describing and disclosing protocols, reagents, and current state of the art technologies that might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to precede such disclosure by virtue of prior invention.

Definitions

The term "biomolecule" refers to a molecule produced by a cell or living organism. Such molecules include, but are not limited to, molecules comprising nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides, polypeptides, proteins, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins). Furthermore, the terms "nucleotide" or polynucleotide" refer to a nucleotide, oligonucleotide, polynucleotide, or any fragment thereof. These phrases also refer to DNA or RNA of genomic or synthetic origin which may be single-stranded or double-stranded and may represent the sense, or the antisense strand, to peptide polynucleotide sequences (i.e. peptide nucleic acids; PNAs), or to any DNA-like or RNA-like material.

The term "fragment" refers to a portion of a polypeptide (parent) sequence that comprises at least 10 consecutive amino acid residues and retains a biological activity and/or some functional characteristics of the parent polypeptide e.g. antigenicity or structural domain characteristics.

The terms "biological sample" and "test sample" refer to all biological fluids and excretions isolated from any given subject. In the context of the invention such samples include, but are not limited to, blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract samples.

The term "specific binding" refers to the binding reaction between a biomolecule and a specific "binding molecule". Related to the invention are binding molecules that include, but are not limited to, proteins, peptides, nucleotides, nucleic acids, hormones, amino acids, sugars, fatty acids, steroids, polynucleotides, carbohydrates, lipids, or a combination thereof (e.g. glycoproteins, ribonucleoproteins, lipoproteins). Furthermore, a binding reaction is considered to be specific when the interaction between said molecules is substantial. In the context of the invention, a binding reaction is considered substantial when the reaction that takes place between said molecules is at least two times the background. Moreover, the term "specific binding conditions" refers to reaction conditions that permit the binding of said molecules such as pH, salt, detergent and other conditions known to those skilled in the art.

The term "interaction" relates to the direct or indirect binding or alteration of biological activity of a biomolecule.

The term "differential diagnosis" refers to a diagnostic decision between a healthy and different disease states, including various stages of a specific disease. A subject is diagnosed as healthy or to be suffering from a specific disease, or a specific stage of a disease based on a set of hypotheses that allow for the distinction between healthy and one or more stages of the disease. The choice between healthy and one or more stages of disease depends on a significant difference between each hypothesis. Under the same principle, a "differential diagnosis" may also refer to a diagnostic decision between one disease type as compared to another (e.g. pancreatic cancer vs. pancreatitis).

The term "pancreatic cancer" refers to a malignant lesion of the pancreas of any given subject, wherein the cancer state is defined according to its stage and/or grade. The various stages of a cancer may be identified using staging systems known to those skilled in the art [e.g. Union Internationale Contre Cancer (UICC) system or American Joint Committee on Cancer (AJC)].

The term "pancreatitis" refers to an inflammation of the pancreas. Pancreatitis is a disease wherein the organ becomes inflamed, and occurs as a spectrum, both in duration and severity as either acute or chronic pancreatitis. The term "acute pancreatitis" refers to a sudden and self-limited inflammation of the pancreas and is often characterised by the onset of abdominal pain resulting from both enzymatic necrosis and said inflammation. The term "chronic inflammation" refers to the process of continuous or relapsing inflammation of the pancreas, typically causing pain and leading to irreversible morphological damage and permanent impairment of function. More, specifically there is a continued loss of pancreatic parenchyma replaced by fibrous tissue. The chief distinctions between acute and chronic pancreatitis are a) whether the pancreas is normal before a symptomatic attack or already chronically damaged and b) whether the inflammation is reversible or not.

The term "healthy individual" refers to a subject possessing good health. Such a subject demonstrates an absence of any disease within the pancreas; preferably an absence of pancreatitis or pancreatic cancer.

The term "precancerous lesion of the pancreas" refers to a biological change within the pancreas such that it becomes susceptible to the development of a cancer. More specifically, a precancerous lesion of the pancreas is a preliminary stage of a pancreatic cancer. Causes of a precancerous lesion may include, but are not limited to, genetic predisposition and exposure to cancer-causing agents (carcinogens); such cancer causing agents include agents that cause genetic damage and induce neoplastic transformation of a cell. Furthermore, the phrase "neoplastic transformation of a cell" refers an alteration in normal cell physiology and includes, but is not limited to, self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

The phrase "differentially present" refers to differences in the quantity of a biomolecule (of a particular apparent molecular mass) present in a sample from a subject as compared to a comparable sample. For example, a biomolecule is present at an elevated level, a decreased level or absent in samples of subjects having pancreatic cancer compared to samples of subjects who do not have a cancer of the pancreas. Therefore in the context of the invention, the term "differentially present biomolecule" refers to the quantity biomolecule (of a particular apparent molecular mass) present within a sample taken from a subject having a disease or cancer of the pancreas as compared to a comparable sample taken from a healthy subject. Within the context of the invention, a biomolecule is differentially present between two samples if the quantity of said biomolecule in one sample is statistically significantly different from the quantity of said biomolecule in another sample.

The term "diagnostic assay" can be used interchangeably with "diagnostic method" and refers to the detection of the presence or nature of a pathologic condition. Diagnostic assays differ in their sensitivity and specificity. Within the context of the invention, the sensitivity of a diagnostic assay is defined as the percentage of diseased subjects who test positive for pancreatitis or pancreatic cancer, and are considered "true positives". Subjects having pancreatitis or a pancreatic cancer, but are not detected by the diagnostic assay are considered "false negatives". Subjects who are not diseased and who test negative in the diagnostic assay are considered "true negatives". Furthermore, the term specificity of a diagnostic assay, as used herein, is defined as 1 minus the false positive rate, where the "false positive rate" is defined as the proportion of those subjects devoid of pancreatitis or a pancreatic cancer but who test positive in said assay.

The term "adsorbent" refers to any material that is capable of accumulating (binding) a biomolecule. The adsorbent typically coats a biologically active surface and is composed of a single material or a plurality of different materials that are capable of binding a biomolecule. Such materials include, but are not limited to, anion exchange materials, cation exchange materials, metal chelators, polynucleotides, oligonucleotides, peptides, antibodies, metal chelators etc.

The term "biologically active surface" refers to any two- or three-dimensional extension of a material that biomolecules can bind to, or interact with, due to the specific biochemical properties of this material and those of the biomolecules. Such biochemical properties include, but are not limited to, ionic character (charge), hydrophobicity, or hydrophilicity.

The term "binding molecule" refers to a molecule that displays an affinity for another molecule. With in the context of the invention such molecules may include, but are not limited to nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polypeptides, carbohydrates, lipids, and combinations thereof (e.g. glycoproteins, ribonucleoproteins, lipoproteins). Preferably, such binding molecules are antibodies.

The term "solution" refers to a homogeneous mixture of two or more substances. Solutions may include, but are not limited to buffers, substrate solutions, elution solutions, wash solutions, detection solutions, standardisation solutions, chemical solutions, solvents, etc. Furthermore, other solutions known to those skilled in the art are also included herein.

The term "mass profile" refers to a mass spectrum as a characteristic property of a given sample or a group of samples. Such a profile, when compared to the mass profile of a second sample or group of samples, will allow for the differentiation between the two samples. In the context of the invention, the mass profile is obtained by treating the biological sample as follows. The sample is diluted it 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% ampholine and subsequently diluted 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5. Thus pre-treated sample is applied to a biologically active surface comprising positively charged quaternary ammonium groups (anion exchange surface) and incubated for 120 minutes. The biomolecules bound to the surface are analysed by gas phase ion spectrometry as described in another section. All but the dilution steps are performed at 20 to 24°C. Dilution steps are performed at 0 to 4°C.

The phrase "apparent molecular mass" refers to the molecular mass value in Dalton (Da) of a biomolecule as it may appear in a given method of investigation, e.g. size exclusion chromatography, gel electrophoresis, or mass spectrometry.

The term "chromatography" refers to any method of separating biomolecules within a given sample such that the original native state of a given biomolecule is retained. Separation of a biomolecule from other biomolecules within a given sample for the purpose of enrichment, purification and/or analysis, may be achieved by methods including, but not limited to, size exclusion chromatography, ion exchange chromatography, hydrophobic and hydrophilic interaction chromatography, metal affinity chromatography, wherein "metal" refers to metal ions (e.g. nickel, copper, gallium, or zinc) of all chemically possible valences, or ligand affinity chromatography wherein "ligand" refers to binding molecules, preferably proteins, antibodies, or DNA. Generally, chromatography uses biologically active surfaces as adsorbents to selectively accumulate certain biomolecules.

The term "mass spectrometry" refers to a method comprising employing an ionization source to generate gas phase ions from a biological entity of a sample presented on a biologically active surface and detecting the gas phase ions with a mass spectrometer.

The phrase "laser desorption mass spectrometry" refers to a method comprising the use of a laser as an ionization source to generate gas phase ions from a biomolecule presented on a biologically active surface and detecting the gas phase ions with a mass spectrometer.

The term "mass spectrometer" refers to a gas phase ion spectrometer that includes an inlet system, an ionisation source, an ion optic assembly, a mass analyser, and a detector.

Within the context of the invention, the terms "detect", "detection" or "detecting" refer to the identification of the presence, absence, or quantity of a biomolecule.

The term "energy absorbing molecule" or "EAM" refers to a molecule that absorbs energy from an energy source in a mass spectrometer thereby enabling desorption of a biomolecule from a biologically active surface. Cinnamic acid derivatives, sinapinic acid and dihydroxybenzoic acid are frequently used as energy-absorbing molecules in laser desorption of biomolecules. See U.S. Pat. No. 5,719,060 (Hutchens & Yip) for a further description of energy absorbing molecules.

The term "training set" refers to a subset of the respective entire available data set. This subset is typically randomly selected, and is solely used for the purpose of classifier construction.

The term "test set" refers to a subset of the entire available data set consisting of those entries not included in the training set. Test data is applied to evaluate classifier performance.

The term "decision tree" refers to a flow-chart-like tree structure employed for classification. Decision trees consist of repeated splits of a data set into subsets. Each split consists of a simple rule applied to one variable, e.g., "if value of 'variable 1' larger than 'threshold 1' then go left else go right". Accordingly, the given feature space is partitioned into a set of rectangles with each rectangle assigned to one class.

The terms "ensemble", "tree ensemble" or "ensemble classifier" can be used interchangeably and refer to a classifier that consists of many simpler elementary classifiers, e.g., an ensemble of decision trees is a classifier consisting of decision trees. The result of the ensemble classifier is obtained by combining all the results of its constituent classifiers, e.g., by majority voting that weights all constituent classifiers equally. Majority voting is especially reasonable in the case of bagging, where constituent classifiers are then naturally weighted by the frequency with which they are generated.

The term "competitor" refers to a variable that can be used as an alternative splitting rule in a decision tree. Within the context of the invention, the competitor is the apparent molecular mass of a given biomolecule. In each step of decision tree construction, only the variable yielding best data splitting is selected. Competitors are non-selected variables with similar but lower performance than the selected variable. They point into the direction of alternative decision trees.

The term "surrogate" refers to a splitting rule that closely mimics the action of the primary split. A surrogate is a variable that can substitute a selected decision tree variable, e.g. in the case of missing values. Not only must a good surrogate split the parent node into descendant nodes similar in size and composition to the primary descendant nodes, it must also match the primary split on the specific cases that go to the left child and right child nodes.

The terms "peak" and "signal" may be used interchangeably and refer to any signal which is generated by a biomolecule when under investigation using a specific method, for example chromatography, mass spectrometry, or any type of spectroscopy like Ultraviolet/Visible Light (UV/Vis) spectroscopy, Fourier Transformed Infrared (FTIR) spectroscopy, Electron Paramagnetic Resonance (EPR) spectroscopy, or Nuclear Mass Resonance (NMR) spectroscopy.

Within the context of the invention, the terms "peak" and "signal" refer to the signal generated by a biomolecule of a certain molecular mass hitting the detector of a mass spectrometer, thus generating a signal intensity which correlates with the amount or concentration of said biomolecule of a given sample. A "peak" and "signal" is defined by two values: an apparent molecular mass value (m/z) and an intensity value generated as described. The mass value is an elemental characteristic of a biological entity, whereas the intensity value accords to a certain amount or concentration of a biological entity with the corresponding apparent molecular mass value, and thus "peak" and "signal" always refer to the properties of this biological entity.

The term "cluster" refers to a signal or peak present in a certain set of mass spectra or mass profiles obtained from different samples belonging to two or more different groups (e.g. cancer and non cancer). Within the set, signals belonging to cluster can differ in their intensities, but not in the apparent molecular masses.

The term "variable" refers to a cluster which is subjected to a statistical analysis aiming towards a classification of samples into two or more different sample groups (e.g. cancer and non cancer) by using decision trees, wherein the sample feature relevant for classification is the intensity value of the variables in the analysed samples.

Detailed Description of the invention

a) Diagnostics

The present invention relates to methods for the differential diagnosis of pancreatitis and/or pancreatic cancers by detecting one or more differentially expressed biomolecules within a test sample of a given subject, comparing results with samples from healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer, or subjects having pancreatitis, wherein the comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer or pancreatitis.

In one aspect of the invention, a method for the differential diagnosis of a pancreatitis and/or pancreatic cancer comprises obtaining a test sample from a given subject, contacting said sample with an adsorbent present on a biologically active surface under specific binding conditions, allowing the biomolecules within the test sample to bind to said adsorbent, detecting one or more bound biomolecules using a detection method, wherein the detection method generates a mass profile of said sample, transforming mass profile data into a computer-readable form comparing the mass profile of said sample with a database containing mass profiles from comparable samples specific for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer, or subjects having pancreatitis. A comparison of mass profiles allows for the medical practitioner to determine if a subject is healthy, has a precancerous lesion of the pancreas, a pancreatic cancer, a metastasised pancreatic cancer or pancreatitis based on the presence, absence or quantity of specific biomolecules.

In more than one embodiment, a single biomolecule or a combination of more than one biomolecule selected from the group having an apparent molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da,

18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da may be detected within a given sample. Detection of a single or a combination of more than one biomolecule of the invention is based on specific sample pre-treatment conditions, the pH of binding conditions, and the type of biologically active surface used for the detection of biomolecules. For example, prior to the detection of the biomolecules described herein, a given sample is pre-treated by diluting 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% ampholine. The denatured sample is then diluted 1:10 in a specific binding buffer (0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5), applied to a biologically active surface comprising of positively-charged quaternary ammonium groups (cationic) and incubated using specific buffer conditions (0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5) to allow for binding of said biomolecules to the above-mentioned biologically active surface.

According to the invention, a biomolecule with the molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da,, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da is detected by diluting the biological sample 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine, and then 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C, applying thus treated sample to a biologically active surface comprising positively charged (cationic) quaternary ammonium groups (anion exchanging), incubating for 120 minutes at 20 to 24°C, and subjecting the bound biomolecules to gas phase ion spectrometry as described in another section.

A biomolecule of the invention may include any molecule that is produced by a cell or living organism, and may have any biochemical property (e.g. phosphorylated proteins, glycosylated proteins, positively charged molecules, negatively charged molecules, hydrophobicity, hydrophilicity), but preferably biochemical properties that allow binding of the biomolecule to a biologically active surface comprising positively charged quaternary ammonium groups after denaturation in 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine and dilution in 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C followed by incubation on said biologically active surface for 120 minutes at 20 to 24°C. Such molecules include, but are not limited to, molecules comprising nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides (DNA or RNA), polypeptides, proteins, antibodies, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins). Preferably a biomolecule may be a nucleotide, polynucleotide, peptide, protein or fragments thereof. Even more preferred are peptide or protein biomolecules or fragments thereof.

The methods for detecting these biomolecules have many applications. For example, a single biomolecule or a combination of more than one biomolecule selected from the group having an apparent molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da,, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da can be measured to differentiate between healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having pancreatic cancer, subjects having a metastasized pancreatic cancer or subjects with pancreatitis, and thus are useful as an aid in the diagnosis of a pancreatic cancer and/or pancreatitis within a subject. Alternatively, said biomolecules may be used to diagnose a subject as healthy.

For example, a biomolecule having the apparent molecular mass of about e.g. 5497 Da is present only in biological samples from patients having a metastasised pancreatic cancer. Mass profiling of two test samples from different subjects, X and Y, reveals the presence of a biomolecule with the apparent molecular mass of about 5497 Da in a sample from test subject X, and the absence of said biomolecule in test sample from subject Y. The medical practitioner is able to diagnose subject X as having a metastasised pancreatic cancer and subject Y as not having a metastasised pancreatic cancer. In yet another example, three biomolecules having the apparent molecular mass of about 1624 Da, 8001 Da and 16986 Da are present in varying quantities in samples specific for precancerous lesions and "early" pancreatic cancers. The biomolecule having the apparent molecular mass of 1624 Da is more present in samples specific for precancerous lesions of the pancreas than for "early" pancreatic cancers. A biomolecule having an apparent molecular mass of 8001 Da is detected in samples from subjects having "early" pancreatic cancers but not in those having a precancerous lesion, whereas the biomolecule having the molecular mass of 16986 Da is present in about the same quantity in both sample types. Such biomolecules are not present in samples from healthy subjects, only those of apparent molecular mass of 4551 Da and 22981Da. Analysis of a test sample reveals the presence of biomolecules having the molecular mass of 16986 Da, 1624 Da and 8001 Da. Comparison of the quantity of the biomolecules within said sample reveals that the biomolecule with an apparent molecular mass of 1624 Da is present at lower levels than those found in samples from subjects having a precancerous lesion. The medical practitioner is able to diagnose the test subject as having an "early" pancreatic cancer. These examples are solely used for the purpose of clarification and are not intended to limit the scope of this invention.

In another aspect of the invention, an immunoassay can be used to determine the presence or absence of a biomolecule within a test sample of a subject. First, the presence or absence of a biomolecule within a sample can be detected using the various immunoassay methods known to those skilled in the art (i.e. ELISA, western blots). If a biomolecule is present in the test sample, it will form an antibody-marker complex with an antibody that specifically binds a biomolecule under suitable incubation conditions. The amount of an antibody-biomolecule complex can be determined by comparing to a standard.

Thus the invention provides a method for the differential diagnosis of pancreatitis and/or pancreatic cancer comprising: detecting of one or more differentially expressed biomolecules within a sample. This method comprises obtaining a test sample from a subject, contacting said sample with a binding molecule specific for a differentially expressed polypeptide, detecting an interaction between the binding molecule and its specific polypeptide, wherein the detection of an interaction indicates the presence or absence of said polypeptide, thereby allowing for the differential diagnosis of a subject as healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer and/or pancreatitis. Binding molecules include, but are not limited to, proteins, peptides, nucleotides, nucleic acids, hormones, amino acids, sugars, fatty acids, steroids, polynucleotides, carbohydrates, lipids, or a combination thereof (e.g. glycoproteins, ribonucleoproteins, lipoproteins), compounds or synthetic molecules. Preferably, binding molecules are antibodies specific for biomolecules selected from the group of having an apparent molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da,, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da.

In another aspect of the invention, a method for detecting the differential presence of one or more biomolecules selected from the group having an apparent molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59

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For example, *in vivo*, antibodies or fragments thereof may be utilised for the detection of a biomolecule in a biological sample comprising: applying a labelled antibody directed against a given biomolecule of the invention to said sample under conditions that favour an interaction between the labelled antibody and its corresponding protein. Depending on the nature of the biological sample, it is possible to determine not only the presence of a biomolecule but also its cellular distribution. For example, in a blood serum sample, only the serum levels of a given biomolecule can be detected, whereas its level of expression and cellular localisation can be detected in histological samples. It will be obvious to those skilled in the art, that a wide variety of methods can be modified in order to achieve such detection.

For example, an antibody coupled to an enzyme is detected using a chromogenic substrate that is recognised and cleaved by the enzyme to produce a chemical moiety, which is readily detected using spectrometric, fluorimetric or visual means. Enzymes used to for labelling include, but are not limited to; malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. Detection may also be accomplished by visual comparison of the extent of the enzymatic reaction of a substrate with that of similarly prepared standards. Alternatively, radiolabelled antibodies can be detected using a gamma or a scintillation counter, or they can be detected using autoradiography. In another example fluorescently labelled antibodies are detected based on the level at which the attached compound fluoresces following exposure to a given wavelength. Fluorescent compounds typically used in antibody labelling include, but are not limited to, fluorescein isothiocynate, rhodamine, phycoerthyrin, phycocyanin, allophycocyani, o-phthaldehyde and fluorescamine. In yet another example, antibodies coupled to a chemi- or bioluminescent compound can be detected by determining the presence of luminescence. Such compounds include, but are not limited to, luminal, isoluminal, theromatic acridinium ester, imidazole, acridinium salt, oxalate ester, luciferin, luciferase and aequorin.

Furthermore, *in vivo* techniques for the detection of a biomolecule of the invention include introducing into a subject a labelled antibody directed against a given polypeptide or fragment thereof.

In more than one embodiment of the invention, the test sample used for the differential diagnosis of a pancreatitis and/or pancreatic cancer of a subject may be of blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract origin. Preferably, test samples are of blood, blood serum, plasma, urine, excreta, prostatic fluid, biopsy, ascites, lymph or tissue extract origin. More preferred are blood, blood serum, plasma, urine, excreta, biopsy, lymph or tissue extract samples. Even more preferred are blood serum, urine, excreta or biopsy samples. Overall preferred are blood serum samples.

Furthermore, test samples used for the methods of the invention are isolated from subjects of mammalian origin, preferably of primate origin. Even more preferred are subjects of human origin.

In addition, the methods of the invention for the differential diagnosis of healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasized pancreatic cancer or subjects having pancreatitis described herein may be combined with other diagnostic methods to improve the outcome of the differential diagnosis. Other diagnostic methods are known to those skilled in the art.

b) Database

In another aspect of the invention, a database comprising of mass profiles specific for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer, or subjects having pancreatitis is generated by contacting biological samples isolated from above-mentioned subjects with an adsorbent on a biologically active surface under specific binding conditions, allowing the biomolecules within said sample to bind said adsorbent, detecting one or more bound biomolecules using a detection method wherein the detection method generates a mass profile of said sample, transforming the mass profile data into a computer-readable form and applying a mathematical algorithm to classify the mass profile as specific for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer or subjects having pancreatitis.

According to the invention, the classification of said mass profiles is performed using the "CART" decision tree approach [classification and regression trees; Breiman et al. (1984) Classification and regression trees. Wadsworth International, Belmont, California] and is known to those skilled in the art. Furthermore, bagging of classifiers is applied to overcome typical instabilities of forward variable selection procedures, thereby increasing overall classifier performance [Breiman L. (1996) Bagging Predictors. Machine learning 24: 123-140].

In more than one embodiment, one or more biomolecules selected from the group having an apparent molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da may be detected within a given biological sample. Detection of said biomolecules of the invention is based on specific sample pre-treatment conditions, the pH of binding conditions, and the type of biologically active surface used for the detection of biomolecules.

Within the context of the invention, biomolecules within a given sample are bound to an adsorbent on a biologically active surface under specific binding conditions, for example, the biomolecules within a given sample are applied to a biologically active surface comprising positively-charged quaternary ammonium groups (cationic) and incubated with 0.1 M Tris-HCl, 0.02% Triton X-100 at a pH of 8.5 to allow for specific binding. Biomolecules that bind to said biologically active surface under these conditions are negatively charged molecules. It should be noted that although the biomolecules of the invention are bound to a cationic adsorbent comprising of positively-charged quaternary ammonium groups, the biomolecules are capable of binding other types of adsorbents, as described in another section using binding conditions known to those skilled in the art. Accordingly, some embodiments of the invention are not limited to the use of cationic adsorbents.

According to the invention, a biomolecule with the molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da is detected by diluting the biological sample 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine, and then 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C, applying thus treated sample to a biologically active surface comprising positively charged (cationic) quaternary ammonium groups (anion exchanging), incubating for 120 minutes at 20 to 24°C, and subjecting the bound biomolecules to gas phase ion spectrometry as described in another section.

In one embodiment of the invention, biological samples used to generate a database of mass profiles for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having a metastasised pancreatic cancer or subjects having pancreatitis, may be of blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract origin. Preferably, biological samples are of blood, blood serum, plasma, urine, excreta, prostatic fluid, biopsy, ascites, lymph or tissue extract origin. More preferred are blood, blood serum, plasma, urine, excreta, biopsy, lymph or tissue extract samples. Even more preferred are blood serum, urine, excreta or biopsy samples. Overall preferred are blood serum samples.

Furthermore, the biological samples related to the invention are isolated from subjects considered to be healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer or having pancreatitis. Said subjects are of mammalian origin, preferably of primate origin. Even more preferred are subjects of human origin.

A subject of the invention that is said to have a precancerous lesion of the pancreas, displays preliminary stages of a cancer, wherein a cell and/or tissue has become susceptible to the development of a cancer as a result of either a genetic predisposition, exposure to a cancer-causing agent (carcinogen) or both.

A genetic pre-disposition may include a predisposition for an autosomal dominant inherited cancer syndrome which is generally indicated by a strong family history of uncommon cancer and/or an association with a specific marker phenotype, a familial cancer (e.g. familial relapsing pancreatitis) wherein an evident clustering of cancer is observed but the role of inherited predisposition may not be clear, or an autosomal recessive syndrome characterised by chromosomal or DNA instability. Cancer-causing agents include agents that stimulate genetic damage and induce neoplastic transformation of a cell. Such agents fall into three categories: 1) chemical carcinogens such as alkylating agents, polycyclic aromatic hydrocarbons, aromatic amines, azo dyes, nitrosamines and amides, asbestos, vinyl chloride, chromium, nickel, arsenic, and naturally occurring carcinogens (e.g. aflotoxin B1); 2) radiation such as ultraviolet (UV) and ionisation radiation including electromagnetic (e.g. x-rays, γ-rays) and particulate radiation (e.g. α and β particles, protons, neutrons); 3) viral and microbial carcinogens such as human Papillomavirus (HPV), Epstein-Barr virus (EBV), hepatitis B virus (HBV), human T-cell leukaemia virus type 1 (HTLV-1), or *Helicobacter pylori*. In addition, environmental factors have also been implicated to play a role in the predisposition of pancreatic cancer. Such factors are known to those skilled in the art and include, but are not limited to smoking, chronic alcohol intake, and the consumption of a high-energy diet rich in fats. Furthermore, pancreatic cancer arises with greater frequency in patient with chronic pancreatitis

Within the context of the invention, cancers of the pancreas (interchangeably referred to as pancreatic cancers within the invention) may also be of various stages, wherein the staging is based on the size of the primary lesion, its extent of spread to regional lymph nodes, and the presence or absence of blood-borne metastases (metastatic pancreatic cancers. The various stages of a cancer may be identified using staging systems known to those skilled in the art [e.g. Union Internationale Contre Cancer (UICC) system or American Joint Committee on Cancer (AJC)]. Also included are different grades of said cancers, wherein the grade of a cancer is based on the degree of differentiation of the epithelial cells within the lining of the pancreas and the number of mitoses as a correlation to a neoplasm's aggression.

A subject said to have pancreatitis displays the condition of an inflammation of the pancreas. Said condition can be characterised as either acute or chronic depending on the severity and duration of the inflammation. Whereas, acute pancreatitis results from the sudden onset of enzymatic necrosis and inflammation of the pancreas, chronic pancreatitis results from repeated pancreatic inflammation over a long period of time combined with the loss of pancreatic parenchyma that is replaced with fibrous tissue). Preferred are subjects with chronic pancreatitis.

Healthy individuals, as related to certain embodiments of the invention, are those that possess good health, and demonstrate an absence of a pancreatic cancer or pancreatitis.

c) Biomolecules

The differential expression of biomolecules in samples from healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having a pancreatic cancer, subjects having metastasised pancreatic cancer, and subjects having pancreatitis, allows for the differential diagnosis of pancreatitis or pancreatic cancer within a subject.

Biomolecules are said to be specific for a particular clinical state (e.g. healthy, precancerous lesion of the pancreas, pancreatic cancer, metastasised pancreatic cancer, pancreatitis) when they are present at different levels within samples taken from subjects in one clinical state as compared to samples taken from subjects from other clinical states (e.g. in subjects with a precancerous lesion of the pancreas vs. in subjects with a metastasised pancreatic cancer). Biomolecules may be present at elevated levels, at decreased levels, or altogether absent within a sample taken from a subject in a particular clinical state (e.g. healthy, precancerous lesion of the pancreas, pancreatic cancer, metastasised pancreatic cancer, pancreatitis). For example, biomolecules A and B are found at elevated levels in samples isolated from healthy subjects as compared to samples isolated from subjects having a precancerous lesion of the pancreas, a pancreatic cancer, a metastatic pancreatic cancer or pancreatitis. Whereas, biomolecules X, Y, Z are found at elevated levels and/or more frequently in samples isolated from subjects having a precancerous lesion of the pancreas as opposed to subjects in good health, having a pancreatic cancer, a metastasised pancreatic cancer or pancreatitis. Biomolecules A and B are said to be specific for healthy subjects, whereas biomolecules X, Y, Z are specific for subjects having a precancerous lesion of the pancreas.

Accordingly, the differential presence of one or more biomolecules found in a test sample compared to samples from healthy subjects, subjects with a precancerous lesion of the pancreas, a pancreatic cancer, a metastasized pancreatic cancer, or pancreatitis, or the mere detection of one or more biomolecules in the test sample provides useful information regarding probability of whether a subject being tested has a precancerous lesion of the pancreas, a pancreatic cancer, a metastasized pancreatic cancer or pancreatitis. The probability that a subject being tested has a precancerous lesion of the pancreas, a pancreatic cancer, a metastasized pancreatic cancer or pancreatitis depends on whether the quantity of one or more biomolecules in a test sample taken from said subject is statistically significantly different from the quantity of one or more biomolecules in a biological sample taken from healthy subjects, subjects having a precancerous lesion of the pancreas, a pancreatic cancer, a metastasised pancreatic cancer, or pancreatitis.

A biomolecule of the invention may be any molecule that is produced by a cell or living organism, and may have any biochemical property (e.g. phosphorylated proteins, positively charged molecules, negatively charged molecules, hydrophobicity, hydrophilicity), but preferably biochemical properties that allow binding of the biomolecule to a biologically active surface comprising positively charged quaternary ammonium groups after denaturation in 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine and dilution in 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C followed by incubation on said biologically active surface for 120 minutes at 20 to 24°C. Such molecules include, but are not limited to, molecules comprising nucleotides, amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides (DNA or RNA), polypeptides, proteins, antibodies, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins). Preferably a biomolecule may be a nucleotide, polynucleotide, peptide, protein or fragments thereof. Even more preferred are peptide or protein biomolecules.

The biomolecules of the invention can be detected based on specific sample pre-treatment conditions, the pH of binding conditions, the type of biologically active surface used for the detection of biomolecules within a given sample and their molecular mass. For example, prior to the detection of the biomolecules described herein, a given sample is pre-treated by diluting 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% ampholine. The denatured sample is then diluted 1:10 in 0.1 M Tris-HCl, 0.02% Triton

X-100, pH 8.5, applied to a biologically active surface comprising positively-charged quaternary ammonium groups (cationic) and incubated using specific buffer conditions (0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5) to allow for binding of said biomolecules to the above-mentioned biologically active surface. It should be noted that although the biomolecules of the invention are detected using a cationic adsorbent positively charged quaternary ammonium groups, as well as specific pre-treatment and binding conditions, the biomolecules are capable of binding other types of adsorbents, as described below, using alternative pre-treatment and binding conditions known to those skilled in the art. Accordingly, some embodiments of the invention are not limited to the use of cationic adsorbents.

The biomolecules of the invention include biomolecules having a molecular mass selected from the group consisting of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da.

According to the invention, a biomolecule with the molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da is detected by diluting the biological sample 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, and 2% Ampholine, and then 1:10 in binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100 at pH 8.5 at 0 to 4°C, applying thus treated sample to a biologically active surface comprising positively charged (cationic) quaternary ammonium groups (anion exchanging), incubating for 120 minutes at 20 to 24°C, and subjecting the bound biomolecules to gas phase ion spectrometry as described in another section.

Although said biomolecules were first identified in blood serum samples, their detection is not limited to said sample type. The biomolecules may also be detected in other samples types, such as blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract. Preferably, samples are of blood, blood serum, plasma, urine, excreta, prostatic fluid, biopsy, ascites, lymph or tissue extract origin. More preferred are blood, blood serum, plasma, urine, excreta, biopsy, lymph or tissue extract samples. Even more preferred are blood serum, urine, excreta or biopsy samples. Overall preferred are blood serum samples.

Since the biomolecules can be sufficiently characterized by their mass and biochemical characteristics such as the type of biologically active surface they bind to or the pH of binding conditions, it is not necessary to identify the biomolecules in order to be able to identify them in a sample. It should be noted that molecular mass and binding properties are characteristic properties of these biomolecules and not limitations on the means of detection or isolation. Furthermore, using the methods described herein, or other methods known in the art, the absolute identity of the markers can be determined. This is important when one wishes to develop and/or screen for specific binding molecules, or to develop a an assay for the detection of said biomolecules using specific binding molecules.

d) Biologically Active Surfaces

In one embodiment of the invention, biologically active surfaces include, but are not restricted to, surfaces that contain adsorbents such as quaternary ammonium groups (anion exchange surfaces), carboxylate groups (cation exchange surfaces), alkyl or aryl chains (hydrophobic interaction, reverse phase chemistry), groups such as nitriloacetic acid that immobilize metal ions such as nickel, gallium, copper, or zinc (metal affinity interaction), or biomolecules such as proteins, preferably antibodies, or nucleic acids, preferably protein binding sequences, covalently bound to the surface via carbonyl diimidazole moieties or epoxy groups (specific affinity interaction). Preferred are adsorbents comprising anion exchange surfaces.

These surfaces may be located on matrices like polysaccharides such as sepharose, e.g. anion exchange surfaces or hydrophobic interaction surfaces, or solid metals, e.g. antibodies coupled to magnetic beads. Surfaces may also include gold-plated surfaces such as those used for Biacore Sensor Chip technology. Other surfaces known to those skilled in the art are also included within the scope of the invention.

Biologically active surfaces are able to adsorb biomolecules like amino acids, sugars, fatty acids, steroids, nucleic acids, polynucleotides, polypeptides, carbohydrates, lipids, and combinations thereof (e.g., glycoproteins, ribonucleoproteins, lipoproteins).

In another embodiment, devices that use biologically active surfaces to selectively adsorb biomolecules may be chromatography columns for Fast Protein Liquid Chromatography (FPLC) and High Pressure Liquid Chromatography (HPLC), where the matrix, e.g. a polysaccharide, carrying the biologically active surface, is filled into vessels (usually referred to as "columns") made of glass, steel, or synthetic materials like polyetheretherketone (PEEK).

In yet another embodiment, devices that use biologically active surfaces to selectively adsorb biomolecules may be metal strips carrying thin layers of the biologically active surface on one or more spots of the strip surface to be used as probes for gas phase ion spectrometry analysis, for example the SAX2 ProteinChip array (Ciphergen Biosystems, Inc.) for SELDI analysis.

e) Mass Profiling

In one embodiment, the mass profile of a sample may be generated using an array-based assay in which the biomolecules of a given sample are bound by biochemical or affinity interactions to an adsorbent present on a biologically active surface located on a solid platform ("array" or "probe"). After the biomolecules have bound to the adsorbent, they are detected using gas phase ion spectrometry. Biomolecules or other substances bound to the adsorbents on the probes can be analyzed using a gas phase ion spectrometer. This includes, e.g., mass spectrometers, ion mobility spectrometers, or total ion current measuring devices. The quantity and characteristics of the biomolecule can be determined using gas phase ion spectrometry. Other substances in addition to the biomolecule of interest can also be detected by gas phase ion spectrometry.

In one embodiment, a mass spectrometer can be used to detect biomolecules on the probe. In a typical mass spectrometer, a probe with a biomolecule is introduced into an inlet system of the mass spectrometer. The biomolecule is then ionized by an ionization source, such as a laser, fast atom bombardment, or plasma. The generated ions are collected by an ion optic assembly, and then a mass analyzer disperses and analyzes the passing ions. Within the scope of this invention, the ionisation course that ionises the biomolecule is a laser.

The ions exiting the mass analyzer are detected by a ion detector. The ion detector then translates information of the detected ions into mass-to-charge ratios. Detection of the presence of a biomolecule or other substances will typically involve detection of signal intensity. This, in turn, can reflect the quantity and character of a biomolecule bound to the probe.

In another embodiment, the mass profile of a sample may be generated using a liquid-chromatography (LC)-based assay in which the biomolecules of a given sample are bound by biochemical or affinity interactions to an adsorbent located in a vessel made of glass, steel, or synthetic material; known to those skilled in the art as a chromatography column. The biomolecules are eluted from the biologically active surface by washing the vessel with appropriate solutions known to those skilled in the art. Such solutions include but are not limited to, buffers, e.g. Tris (hydroxymethyl) aminomethane hydrochloride (TRIS-HCl), buffers containing salt, e.g. sodium chloride (NaCl), or organic solvents, e.g. acetonitrile. Biomolecule mass profiles are generated by application of the eluting biomolecules of the sample by direct connection via an electrospray device to a mass spectrometer (LC/ESI-MS).

Conditions that promote binding of biomolecules to an adsorbent are known to those skilled in the art (reference) and ordinarily include parameters such as pH, the concentration of salt, organic solvent, or other competitors for binding of the biomolecule to the adsorbent. Within the scope of the invention, incubation temperatures are of at least 0 to 100°C, preferably of at least 4 to 60°C, and most preferably of at least 15 to 30°C. Varying additional parameters, such as incubation time, the concentration of detergent, e.g., 3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS), or reducing agents, e.g. dithiothreitol (DTT), are also known to those skilled in the art. Various degrees of binding can be accomplished by combining the above stated conditions as needed, and will be readily apparent to those skilled in the art.

f) Methods for detecting biomolecules within a sample

In yet another aspect, the invention relates to methods for detecting differentially present biomolecules in a test sample and/or biological sample. Within the context of the invention, any suitable method can be used to detect one or more of the biomolecules described herein. For example, gas phase ion spectrometry can be used. This technique includes, e.g., laser desorption/ionization mass spectrometry. Preferably, the test and/or biological sample is prepared prior to gas phase ion spectrometry, e.g., pre-fractionation, two-dimensional gel chromatography, high performance liquid chromatography, etc. to assist detection of said biomolecules. Detection of said biomolecules can also be achieved using methods other than gas phase ion spectrometry. For example, immunoassays can be used to detect the biomolecules within a sample.

In one embodiment, the test and/or biological sample is prepared prior to contacting a biologically active surface and is in aqueous form. Examples said samples include, but are not limited to, blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, tears, saliva, sweat, ascites, cerebrospinal fluid, milk, lymph, or tissue extract samples. Furthermore, solid test and/or biological samples, such as excreta or biopsy samples can be solubilised in or admixed with an eluent using methods known to those skilled in the art such that said samples may be easily applied to a biologically active surface. Test and/or biological samples in the aqueous form can be further prepared using specific solutions for denaturation (pre-treatment) like sodium dodecyl sulphate (SDS), mercaptoethanol, urea, etc. For example, a test and/or biological sample of the invention can be denatured prior to contacting a biologically active surface comprising of quaternary ammonium groups by diluting said sample 1:5 with a buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 2% ampholine.

The sample is contacted with a biologically active surface using any techniques including bathing, soaking, dipping, spraying, washing over, or pipetting, etc. Generally, a volume of sample containing from a few atomoles to 100 picomoles of a biomolecule in about 1 to 500 µl is sufficient for detecting binding of the biomolecule to the adsorbent.

The pH value of the solvent in which the sample contacts the biologically active surface is a function of the specific sample and the selected biologically active surface. Typically, a sample is contacted with a biologically active surface under pH values between 0 and 14, preferably between about 4 and 10, more preferably between 4.5 and 9.0, and most preferably, at pH 8.5. The pH value depends on the type of adsorbent present on a biologically active surface and can be adjusted accordingly.

The sample can contact the adsorbent present on a biologically active for a period of time sufficient to allow the marker to bind to the adsorbent. Typically, the sample and the biologically active surface are contacted for a period of between about 1 second and about 12 hours, preferably, between about 30 seconds and about 3 hours, and most preferably for 120 minutes.

The temperature at which the sample contacts the biologically active surface (incubation temperature) is a function of the specific sample and the selected biologically active surface. Typically, the washing solution can be at a temperature of between 0 and 100°C, preferably between 4 and 37°C, and most preferably between 20 and 24°C.

For example, a biologically active surface comprising of quaternary ammonium groups (anion exchange surface) will bind the biomolecules described herein when the pH value is between 6.5 and 9.0. Optimal binding of the biomolecules of the present invention occurs at a pH of 8.5. Furthermore, a sample is contacted with said biologically active surface for 120 minutes at a temperature of 20 – 24 °C.

Following contacting a sample or sample solution with a biological surface, it is preferred to remove any unbound biomolecules so that only the bound biomolecules remain on the biologically active surface. Washing unbound biomolecules are removed by methods known to those skilled in the art such as bathing, soaking, dipping, rinsing, spraying, or washing the biologically active surface with an eluent or a washing solution. A microfluidics process is preferably used when a washing solution such as an eluent is introduced to small spots of adsorbents on the biologically active surface. Typically, the washing solution can be at a temperature of between 0 and 100°C, preferably between 4 and 37°C, and most preferably between 20 and 24°C.

Washing solution or eluents used to wash the unbound biomolecules from a biologically active surface include, but are not limited to, organic solutions, aqueous solutions such as buffers wherein a buffer may contain detergents, salts, or reducing agents in appropriate concentrations as those known to those skilled in the art.

Aqueous solutions are preferred for washing biologically active surfaces. Exemplary aqueous solutions include, but not limited to, HEPES buffer, Tris buffer, phosphate buffered saline (PBS), and modifications thereof. The selection of a particular washing solution or an eluent is dependent on other experimental conditions (e. g., types of adsorbents used or biomolecules to be detected), and can be determined by those of skill in the art. For example, if a biologically active surface comprising a quaternary ammonium group as adsorbent (anion exchange surface) is used, then an aqueous solution, such as a Tris buffer, may be preferred. In another example, if a biologically active surface comprising a carboxylate group as adsorbent (cation exchange surface) is used, then an aqueous solution, such as an acetate buffer, may be preferred.

Optionally, an energy absorbing molecule (EAM), e.g. in solution, can be applied to biomolecules or other substances bound on the biologically active surface by spraying, pipetting or dipping. Applying an EAM can be done after unbound materials are washed off of the biologically active surface. Exemplary energy absorbing molecules include, but are not limited to, cinnamic acid derivatives, sinapinic acid and dihydroxybenzoic acid.

Once the biologically active surface is free of any unbound biomolecules, adsorbent-bound biomolecules are detected using gas phase ion spectrometry. The quantity and characteristics of a biomolecule can be determined using said method. Furthermore, said biomolecules can be analyzed using a gas phase ion spectrometer such as mass spectrometers, ion mobility spectrometers, or total ion current measuring devices. Other gas phase ion spectrometers known to those skilled in the art are also included.

In one embodiment, mass spectrometry can be used to detect biomolecules of a given sample present on a biologically active surface. Such methods include, but are not limited to, matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF), surface-enhanced laser desorption ionization/time-of-flight (SELDI-TOF), liquid chromatography coupled with MS, MS-MS, or ESI-MS. Typically, biomolecules are analysed by introducing a biologically active surface containing said biomolecules, ionizing said biomolecules to generate ions that are collected and analysed.

In a preferred embodiment, the biomolecules present in a sample are detected using gas phase ion spectrometry, and more preferably, using mass spectrometry. In one embodiment, matrix-assisted laser desorption/ionization ("MALDI") mass spectrometry can be used. In MALDI, the sample is partially purified to obtain a fraction that essentially consists of a marker using separation methods such as two-dimensional gel electrophoresis or high performance liquid chromatography (HPLC).

In another embodiment, surface-enhanced laser desorption/ionization mass spectrometry ("SELDI") can be used. SELDI uses a substrate comprising adsorbents to capture biomolecules, which can then be directly desorbed and ionized from the substrate surface during mass spectrometry. Since the substrate surface in SELDI captures biomolecules, a sample need not be partially purified as in MALDI. However, depending on the complexity of a sample and the type of adsorbents used, it may be desirable to prepare a sample to reduce its complexity prior to SELDI analysis.

For example, biomolecules bound to a biologically active surface can be introduced into an inlet system of the mass spectrometer. The biomolecules are then ionized by an ionization source such as a laser, fast atom bombardment, or plasma. The generated ions are then collected by an ion optic assembly, and then a mass analyzer disperses the passing ions. The ions exiting the mass analyzer are detected by a detector and translated into mass-to-charge ratios. Detection of the presence of a biomolecule typically involves detection of its specific signal intensity, and reflects the quantity and character of said biomolecule.

In a preferred embodiment, a laser desorption time-of-flight mass spectrometer is used with the probe of the present invention. In laser desorption mass spectrometry, biomolecules bound to a biologically active surface are introduced into an inlet system. Biomolecules are desorbed and ionized into the gas phase by a laser. The ions generated are then collected by an ion optic assembly. These ions are accelerated through a short high-voltage field and allowed to drift into a high vacuum chamber of a time-of-flight mass analyzer. At the far end of the high vacuum chamber, the accelerated ions collide with a detector surface at a different time. Since the time-of-flight is a function of the mass of the ions, the elapsed time between ionization and impact can be used to identify the presence or absence of molecules of a specific mass.

The detection of biomolecules described herein can be enhanced using certain selectivity conditions (e. g., types of adsorbents used or washing solutions). In a preferred embodiment, the same or substantially the same selectivity conditions that were used to discover the biomolecules can be used in the methods for detecting a biomolecule in a sample.

Combinations of the laser desorption time-of-flight mass spectrometer with other components described herein, in the assembly of mass spectrometer that employs various means of desorption, acceleration, detection, measurement of time, etc., are known to those skilled in the art.

Data generated by desorption and detection of markers can be analyzed with the use of a programmable digital computer. The computer program generally contains a readable medium that stores codes. Certain codes can be devoted to memory that include the location of each feature on a biologically active surface, the identity of the adsorbent at that feature and the elution conditions used to wash the adsorbent. Using this information, the program can then identify the set of features on the biologically active surface defining certain selectivity characteristics (e. g. types of adsorbent and eluents used). The computer also contains codes that receive as data (input) on the strength of the signal at various molecular masses received from a particular addressable location on the biologically active surface. This data can indicate the number of biomolecules detected, as well as the strength of the signal and the determined molecular mass for each biomolecule detected.

Data analysis can include the steps of determining signal strength (e. g., height of peaks) of a biomolecule detected and removing "outliers" (data deviating from a predetermined statistical distribution). For example, the observed peaks can be normalized, a process whereby the height of each peak relative to some reference is calculated. For example, a reference can be background noise generated by instrument and chemicals (e. g., energy absorbing molecule), which is set as zero in the scale. Then the signal strength detected for each biomolecule can be displayed in the form of relative intensities in the scale desired (e. g., 100). Alternatively, a standard may be admitted with the sample so that a peak from the standard can be used as a reference to calculate relative intensities of the signals observed for each biomolecule or other biomolecules detected.

The computer can transform the resulting data into various formats for displaying. In one format, referred to as "spectrum view", a standard spectral view can be displayed, wherein the view depicts the quantity of a biomolecule reaching the detector at each particular molecular mass. In another format, referred to as "scatter plot" only the peak height and mass information are retained from the spectrum view, yielding a cleaner image and enabling biomolecules with nearly identical molecular mass to be more visible.

Using any of the above display formats, it can be readily determined from the signal display whether a biomolecule having a particular molecular mass is detected from a sample. Preferred biomolecules of the invention are biomolecules with an apparent molecular mass of about 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da. Moreover, from the strength of signal, the amount of a biomolecule bound on the biologically active surface can be determined.

g) Identification of proteins

In the event that the biomolecules of the invention are proteins, the present invention comprises a method for the identification of these proteins, especially by obtaining their amino acid sequence. This method comprises the purification of said proteins from the complex biological sample (blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, tears, saliva, sweat, ascites, cerebrospinal fluid, milk, lymph, or tissue extract samples) by fractionating said sample using techniques known by the one of ordinary skill in the art, most preferably protein chromatography (FPLC, HPLC).

The biomolecules of the invention include those proteins with a molecular mass selected from 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da.

Furthermore, the method comprises the analysis of the fractions for the presence and purity of said proteins by the method which was used to identify them as differentially expressed biomolecules, for example two-dimensional gel electrophoresis, SELDI mass spectrometry of MALDI mass spectrometry, but most preferably MALDI mass spectrometry. The method also comprises an analysis of the purified proteins aiming towards the revealing of their amino acid sequence. This analysis may be performed using techniques in mass spectroscopy known to those skilled in the art.

In one embodiment, this analysis may be performed using peptide mass fingerprinting, revealing information about the specific peptide mass profile after proteolytic digestion of the investigated protein.

In another embodiment, this analysis may be preferably performed using post-source-decay (PSD), or ESI-MS, but most preferably ESI-MS, revealing mass information about all possible fragments of the investigated protein or proteolytic peptides thereof leading to the amino acid sequence of the investigated protein of proteolytic peptide thereof.

The information revealed by the aforementioned techniques can be used to feed world-wide-web search engines, such as MS Fit (Protein Prospector, http://prospector.ucsf.edu) for information obtained from peptide mass fingerprinting, or MS Tag (Protein Prospector, http://prospector.ucsf.edu) for information obtained from PSD, or mascot (www.matrixscience.com) for information obtained from MSMS and peptide mass fingerprinting, for the alignment of the obtained results with data available in public protein sequence databases, such as SwissProt (http://us.expasy.org/sprot/), NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), EMBL (http://srs.embl-heidelberg.de:8000/srs5/) which leads to a confident information about the identity of said proteins.

This information may comprise, if available, the complete amino acid sequence, the calculated molecular mass, the structure, the enzymatic activity, the physiological function, and gene expression of the investigated proteins.

h) Kits

In yet another aspect, the invention provides kits using the methods of the invention as described in the section Diagnostics for the differential diagnosis of pancreatitis or pancreatic cancer, wherein the kits are used to detect the biomolecules of the present invention.

The methods used to detect the biomolecules of the invention can also be used to determine whether a subject is at risk of developing pancreatitis or pancreatic cancer, or has developed pancreatitis or pancreatic cancer. Such methods may also be employed in the form of a diagnostic kit comprising an antibody specific to a biomolecule of the invention or a biologically active surface described herein, which may be conveniently used, for example, in clinical settings to diagnose patients exhibiting symptoms or a family history of a non-steroid dependent cancer. Such diagnostic kits also include solutions and materials necessary for the detection of a biomolecule of the invention, and instructions to use the kit based on the above-mentioned methods.

The biomolecules of the invention include those proteins with a molecular mass selected from 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558 Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da.

For example, the kits can be used to detect one or more of differentially present biomolecules as described above in a test sample of subject. The kits of the invention have many applications. For example, the kits can be used to differentiate if a subject is healthy, having a precancerous lesion of the pancreas, a pancreatic cancer, a metastasized pancreatic cancer or pancreatitis. Thus aiding the diagnosis of pancreatitis or pancreatic cancer. In another example, the kits can be used to identify compounds that modulate expression of said biomolecules.

In one embodiment, a kit comprises an adsorbent on a biologically active surface, wherein the adsorbent is suitable for binding one or more biomolecules of the invention, a denaturation solution for the pre-treatment of a sample, a binding solution, a washing solution or instructions for making a denaturation solution, binding solution, or washing solution, wherein the combination allows for the detection of a biomolecule using gas phase ion spectrometry. Such kits can be prepared from the materials described in other previously detailed sections (e. g., denaturation buffer, binding buffer, adsorbents, washing solutions, etc.).

In some embodiments, the kit may comprise a first substrate comprising an adsorbent thereon (e. g., a particle functionalized with an adsorbent) and a second substrate onto which the first substrate can be positioned to form a probe, which is removably insertable into a gas phase ion spectrometer. In other embodiments, the kit may comprise a single substrate, which is in the form of a removably insertable probe with adsorbents on the substrate.

In another embodiment, a kit comprises a binding molecule that specifically binds to a biomolecule related to the invention, a detection reagent, appropriate solutions and instructions on how to use the kit. Such kits can be prepared from the materials described above, and other materials known to those skilled in the art. A binding molecule used within such a kit may include, but is not limited to, proteins, peptides, nucleotides, nucleic acids, hormones, amino acids, sugars, fatty acids, steroids, polynucleotides, carbohydrates, lipids, or a combination thereof (e.g. glycoproteins, ribonucleoproteins, lipoproteins), compounds or synthetic molecules. Preferably, a binding molecule used in said kit is an antibody.

In either embodiment, the kit may optionally further comprise a standard or control information so that the test sample can be compared with the control information standard to determine if the test amount of a marker detected in a sample is a diagnostic amount consistent with a diagnosis of pancreatitis or pancreatic cancer.

Each recorded measurement reading is accompanied by a margin of deviation. The latter statistical imprecision is well-known to those skilled in the art. In the scope of the present invention, the margin of deviation is exclusively device-specific. That means it is caused by the type of analytical device used which is preferably a mass spectrometer. The accuracy of the recorded measurement reading is specified by a fixed percentage. In the meaning of the present invention, each disclosed molecular mass represents the averaged value of that range which deviates from the averaged value about ± 0.5 %. Furthermore, slight differences appear in the molecular mass value itself which concerns the same protein in parallel patent applications disclosing the matter of cancer biomarkers. There are three reasons to be considered. First, each molecular mass results from the analysis of samples belonging to another type of cancer. The origin of sample, the cellular status, the environmental conditions of the gathered tissue etc. exert an influence on the measurements. Secondly, the given molecular mass of the biomarkers represents the averaged value which is calculated from the data of numerous samples of each cancer species. Thirdly, measuring errors might be also imaginable, for example due to the sample preparation.

Above statements are further illustrated by examples which should not be construed as limiting with regard to the type of disease, the number of given molecular masses or in any other way. The following molecular masses of biomolecules are regarded as equivalent:

(i) 2020 ± 10 (epithelial cancer), 2020 ± 10 (colorectal cancer) and 2020 ± 10 (pancreatic cancer)
(ii) 2270 ± 11 (colorectal cancer) and 2271 ± 11 (pancreatic cancer)
(iii) 3946 ± 20 (epithelial cancer), 3946 ± 20 (colorectal cancer) and 3951 ± 20 (pancreatic cancer)
(iv) 4104 ± 21 (epithelial cancer), 4103 ± 21 (colorectal cancer) and 4108 ± 21 (pancreatic cancer)
(v) 4154 ± 21 (epithelial cancer) and 4154 ± 21 (pancreatic cancer)
(vi) 4242 ± 21 (colorectal cancer) and 4249 ± 21 (pancreatic cancer)
(vii) 4298 ± 21 (epithelial cancer), 4295 ± 21 (colorectal cancer) and 4307 ± 21 (pancreatic cancer)
(viii) 4360 ± 22 (epithelial cancer), 4359 ± 22 (colorectal cancer) and 4364 ± 22 (pancreatic cancer)
(ix) 4477 ± 22 (epithelial cancer), 4476 ± 22 (colorectal cancer) and 4480 ± 22 (pancreatic cancer)
(x) 4546 ± 23 (colorectal cancer) and 4551 ± 23 (pancreatic cancer)
(xi) 4607 ± 23 (colorectal cancer) and 4614 ± 23 (pancreatic cancer)
(xii) 4719 ± 24 (colorectal cancer) and 4725 ± 24 (pancreatic cancer)
(xiii) 4830 ± 24 (colorectal cancer) and 4836 ± 24 (pancreatic cancer)
(xiv) 4867 ± 24 (epithelial cancer), 4865 ± 24 (colorectal cancer) and 4875 ± 24 (pancreatic cancer)
(xv) 4958 ± 25 (epithelial cancer), 4963 ± 25 (colorectal cancer) and 4969 ± 25 (pancreatic cancer)

(xvi) 5112 ± 26 (colorectal cancer) and 5119 ± 26 (pancreatic cancer)

(xvii) 5491 ± 27 (epithelial cancer), 5493 ± 27 (colorectal cancer) and 5497 ± 27 (pancreatic cancer)

(xviii) 5650 ± 28 (epithelial cancer), 5648 ± 28 (colorectal cancer) and 5657 ± 28 (pancreatic cancer)

(xix) 5854 ± 29 (colorectal cancer) and 5857 ± 29 (pancreatic cancer)

(xx) 6449 ± 32 (epithelial cancer), 6446 ± 32 (colorectal cancer) and 6458 ± 32 (pancreatic cancer)

(xxi) 6876 ± 34 (epithelial cancer), 6852 ± 34 (colorectal cancer) and 6866 ± 34 (pancreatic cancer)

(xxii) 6897 ± 35 (colorectal cancer) and 6908 ± 35 (pancreatic cancer)

(xxiii) 7001 ± 35 (epithelial cancer), 6999 ± 35 (colorectal cancer) and 7013 ± 35 (pancreatic cancer)

(xxiv) 7657 ± 38 (colorectal cancer) and 7637 ± 38 (pancreatic cancer)

(xxv) 7969 ± 40 (epithelial cancer) and 8001 ± 40 (pancreatic cancer)

(xxvi) 8232 ± 41 (epithelial cancer), 8215 ± 41 (colorectal cancer) and 8237 ± 41 (pancreatic cancer)

(xxvii) 8474 ± 42 (colorectal cancer) and 8494 ± 42 (pancreatic cancer)

(xxviii) 8574 ± 43 (colorectal cancer) and 8596 ± 43 (pancreatic cancer)

(xxix) 8711 ± 44 (epithelial cancer), 8702 ± 44 (colorectal cancer) and 8717 ± 44 (pancreatic cancer)

(xxx) 8780 ± 44 (colorectal cancer) and 8794 ± 44 (pancreatic cancer)

(xxxi) 8922 ± 45 (colorectal cancer) and 8942 ± 45 (pancreatic cancer)

(xxxii) 9078 ± 45 (colorectal cancer) and 9099 ± 45 (pancreatic cancer)

(xxxiii) 9143 ± 46 (colorectal cancer) and 9163 ± 46 (pancreatic cancer)

(xxxiv) 9201 ± 46 (colorectal cancer) and 9220 ± 46 (pancreatic cancer)

(xxxv) 9359 ± 47 (colorectal cancer) and 9382 ± 47 (pancreatic cancer)

(xxxvi) 9425 ± 47 (colorectal cancer) and 9443 ± 47 (pancreatic cancer)

(xxxvii) 9581 ± 48 (colorectal cancer) and 9604 ± 48 (pancreatic cancer)

(xxxviii) 9641 ± 48 (colorectal cancer) and 9652 ± 48 (pancreatic cancer)

(xxxix) 9718 ± 49 (colorectal cancer) and 9741 ± 49 (pancreatic cancer)

(xl) 10215 ± 51 (colorectal cancer) and 10233 ± 51 (pancreatic cancer)

(xli) 10440 ± 52 (colorectal cancer) and 10455 ± 52 (pancreatic cancer)

(xlii) 10665 ± 53 (epithelial cancer) and 10748 ± 54 (pancreatic cancer)
(xliii) 11216 ± 56 (colorectal cancer) and 11241 ± 56(pancreatic cancer)
(xliv) 11464 ± 57 (colorectal cancer) and 11488 ± 57 (pancreatic cancer)
(xlv) 11547 ± 58 (colorectal cancer) and 11558 ± 58 (pancreatic cancer)
(xlvi) 11693 ± 58 (colorectal cancer) and 11713 ± 58 (pancreatic cancer)
(xlvii) 12669 ± 63 (epithelial cancer), 12619 ± 63 (colorectal cancer) and 12648 ± 63 (pancreatic cancer)
(xlviii) 13784 ± 69 (colorectal cancer) and 13800 ± 69 (pancreatic cancer)
(xlix) 14798 ± 74 (colorectal cancer) and 14829 ± 74 (pancreatic cancer)
(l) 15140 ± 76 (colorectal cancer) and 15168 ± 76 (pancreatic cancer)
(li) 15350 ± 77 (colorectal cancer) and 15378 ± 77 (pancreatic cancer)
(lii) 15879 ± 79 (colorectal cancer) and 15858 ± 79 (pancreatic cancer)
(liii) 15959 ± 80 (epithelial cancer), 15957 ± 80 (colorectal cancer) and 15984 ± 80 (pancreatic cancer)
(liv) 16104 ± 81 (colorectal cancer) and 16141 ± 81 (pancreatic cancer)
(lv) 16164 ± 81 (epithelial cancer), 16164 ± 81 (colorectal cancer) and 16200 ± 81 (pancreatic cancer)
(lvi) 16953 ± 85 (colorectal cancer) and 16986 ± 85 (pancreatic cancer)
(lvii) 17406 ± 87 (epithelial cancer), 17397 ± 87 (colorectal cancer) and 17426 ± 87 (pancreatic cancer)
(lviii) 17890 ± 89 (colorectal cancer) and 17932 ± 89 (pancreatic cancer)
(lix) 18133 ± 91 (epithelial cancer), 18115 ± 91 (colorectal cancer) and 18153 ± 91 (pancreatic cancer)
(lx) 18390 ± 92 (colorectal cancer) and 18424 ± 92 (pancreatic cancer)
(lxi) 22951 ± 115 (colorectal cancer) and 22981 ± 115 (pancreatic cancer)
(lxii) 28055 ± 140 (colorectal cancer) and 28009 ± 140 (pancreatic cancer)
(lxiii) 28259 ± 141 (colorectal cancer) and 28124 ± 141 (pancreatic cancer)

In all examples, each recorded measurement reading is overlapping with any others within its margin of deviation.

A further calculation of averaged values which incorporates the matching molecular masses of each type of cancer is known to those skilled in the art. By applying formulas which the method of error calculation by means of weights (weighted average) is based upon, the following generalized results are obtained for the aforementioned examples:

(i) 2020 ± 10
(ii) 2271 ± 11
(iii) 3948 ± 20
(iv) 4105 ± 21
(v) 4153 ± 21
(vi) 4246 ± 21
(vii) 4300 ± 21
(viii) 4361 ± 22
(ix) 4478 ± 22
(x) 4549 ± 23
(xi) 4611 ± 23
(xii) 4722 ± 24
(xiii) 4833 ± 24
(xiv) 4869 ± 24
(xv) 4963 ± 25
(xvi) 5116 ± 26
(xvii) 5494 ± 27
(xviii) 5652 ± 28
(xix) 5856 ± 29
(xx) 6451 ± 32
(xxi) 6865 ± 34
(xxii) 6903 ± 35
(xxiii) 7004 ± 35
(xxiv) 7647 ± 38
(xxv) 7985 ± 40
(xxvi) 8228 ± 41
(xxvii) 8484 ± 42
(xxviii) 8585 ± 43
(xxix) 8710 ± 44
(xxx) 8787 ± 44

(xxxi) 8932 ± 45

(xxxii) 9089 ± 45

(xxxiii) 9153 ± 46

(xxxiv) 9211 ± 46

(xxxv) 9371 ± 47

(xxxvi) 9434 ± 47

(xxxvii) 9593 ± 48

(xxxviii) 9647 ± 48

(xxxix) 9730 ± 49

(xl) 10224 ± 51

(xli) 10448 ± 52

(xlii) 10707 ± 53

(xliii) 11229 ± 56

(xliv) 11476 ± 57

(xlv) 11553 ± 58

(xlvi) 11703 ± 58

(xlvii) 12645 ± 63

(xlviii) 13792 ± 69

(xlix) 14814 ± 74

(l) 15154 ± 76

(li) 15364 ± 77

(lii) 15869 ± 79

(liii) 15967 ± 80

(liv) 16123 ± 81

(lv) 16176 ± 81

(lvi) 16970 ± 85

(lvii) 17410 ± 87

(lviii) 17911 ± 89

(lix) 18134 ± 91

(lx) 18407 ± 92

(lxi) 22966 ± 115

(lxii) 28032 ± 140

(lxiii) 28192 ± 141

The present invention is further illustrated by the following examples, which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications), as cited throughout this application, are hereby expressly incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are known to those skilled in the art. Such techniques are explained fully in the literature.

Example 1. Sample collection for pancreatitis evaluation.

Serum samples were obtained from a total of 158 individuals, which included two different groups of subjects. In the first group (group I), sera were drawn from 79 pancreatic cancer patients, undergoing diagnosis and treatment of pancreatic cancer at the Department of Gastroenterology and Surgery of the University of Magdeburg (Germany). Serum samples were collected from the patients directly before surgery. At this time, a primary diagnosis was made based on standard techniques e.g. ultrasonic testing, ERCP or CT, and/or other means for the detection of diseases of the pancreas. In most cases the final diagnosis was confirmed by histological evaluation after surgery. In about 30% of the cases surgery was not possible due to the advanced stage of cancer. Follow-up data for all pancreatic cancer patients are currently collected and will be available for later studies.

The non-cancer control group (group II) consisted of 79 subjects with a non-malignant disease of the pancreas (chronic inflammation of the pancreas), which were recruited from the Department of Gastroenterology and Surgery of the University Hospital in Magdeburg (Germany). Serum samples were collected before surgery. At this time, a primary diagnosis was made based on standard techniques e.g. ultrasonic testing, ERCP or CT, and/or other means for the detection of diseases of the pancreas. In all cases the final diagnosis was confirmed by histological evaluation after surgery. Follow-up data for all non-cancer controls are currently collected and will be available for later studies.

Example 2. ProteinChip Array analysis.

ProteinChip Arrays of the SAX2-type (strong anion exchanger) were arranged into a bioprocessor (Ciphergen Biosystems, Inc.), a device that contains up to 12 ProteinChips and facilitates processing of the ProteinChips. The ProteinChips were pre-incubated in the bioprocessor with 200 µl binding buffer (0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5) for two times 15 minutes. 10 µl of serum sample was diluted 1:5 in a buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholine) and again diluted 1:10 in the binding buffer. Then, 300 µl of this mixture (equivalent to 6 µl original serum sample) were directly applied onto the spots of the SAX2 ProteinChips. In between dilution steps and prior to the application to the spots, the sample was kept on ice (at 0°C). After incubation for 120 minutes at 20 to 24 °C the chips were incubated with 200 µl binding buffer, before 2 x 0.5 µl EAM solution (20 mg/ml sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) was applied to the spots. After air-drying for 10 min, the ProteinChips were placed in the ProteinChip Reader (ProteinChip Biology System II, Ciphergen Biosystems, Inc.) and time-of-flight spectra were generated by laser shots collected in the positive mode at an average laser intensity of 215, with the detector sensitivity of 8. Sixty laser shots per average spectra were performed.

Calibration of mass accuracy was performed by using the following mixture of mass standard calibrant proteins: Dynorphin A (porcine, 209 – 225, 2147.50 Da), Beta-endorphin (human, 61 – 91, 3465.00 Da), Insulin (bovine, 5733.58 Da), and Cytochrome c (bovine, 12230.90 Da) at a concentration of 1.21 pmol/µl, and Myoglobin (equine cardiac, 16951.50 Da) at a concentration of 5.16 pmol/µl. 0.5µl of this mixture was applied to a single spot of a H4 ProteinChip array. After air-drying of the drop, 2 x 1 µl matrix solution (a saturated solution of sinapinic acid in 50% acetonitrile 0.5% trifluoracetic acid) was applied to the spot. The drop was allowed to air-dry for 10 min after each application of matrix solution.

The ProteinChip was placed in the ProteinChip Reader (Biology System II, Ciphergen Biosystems, Inc.) and time-of-flight spectra were generated by laser shots collected in the positive mode at laser intensity 210, with the detector sensitivity of 8. Sixty laser shots per average spectra were performed. Subsequently, Time-Of-Flight values were correlated to the molecular masses of the standard proteins, and calibration was performed according to the instrument manual.

Example 3. Peak detection and data analysis.

The analysis of the data was performed by automatic peak detection and alignment using the operating software of the ProteinChip Biology System II, the ProteinChip Software Version 3.1 (Ciphergen Biosystems, Inc.). Figure 1 shows a comparison of protein mass spectra detected using the above mentioned SAX2 ProteinChip arrays for samples isolated from patients suffering from chronic pancreatitis (C1 and C2) and of patients with pancreatic cancer (T1 and T2).

The complete set of patients was randomly divided into a training set and a test set. The train set comprised of 53 randomly selected patients with pancreatic cancer and 53 randomly selected patients with pancreatitis. The test set comprised of 26 randomly selected patients with pancreatic cancer and 26 randomly selected patients with pancreatitis.

The m/z values of all mass spectra selected for the analysis ranged between 1500 Da and 30000 Da, wherein smaller masses were not used since artefacts with the "Energy Absorbing Molecule, EAM" ("Matrix") could not be excluded, and higher masses were not detected under the chosen experimental conditions. First, baseline subtraction was conducted for each mass spectrum followed by normalisation of the spectra according to the intensity of the total ion current. Thirdly, automatic peak detection was applied as previously described by Adam et al., using the "Biomarker Wizard" tool of the ProteinChip Software Version 3.1 (Ciphergen Biosystems, Inc.). The following settings were chosen for peak detection by "Biomarker Wizard": a) auto-detect peaks to cluster, b) first pass: 5 signal/noise, c) minimum peak threshold: 5% of all spectra, d) deletion of user-detected peaks below threshold, e) cluster mass window: +/- 0.3% of mass, f) second pass: 2 signal/noise. Using these settings, 81 signal clusters were identified.

The normalization coefficient generated by normalizing the spectra of the train sets and the cluster information of the train sets generated by the "Biomarker Wizard" tool of the software were saved and used to externally normalize the spectra of the corresponding test sets and to cluster the signals of the corresponding test sets according to the normalization and peak identification of the train sets.

The cluster information for each train and test set (containing sample ID and sample group, cluster mass values and cluster signal intensities for each spectrum within the sets) was transformed into an interchangeable data format (a .csv table) using the "Sample group statistics" function of the "Biomarker Wizard" tool of the ProteinChip Software Version 3.1. In this format, the data can be analyzed by a specific software for the generation of regression and classification trees (see examples 4 to 6).

Example 4. Construction of classifiers.

Two classifiers with binary target variable (cancer versus non-cancer) were constructed: First, as a proof of principle, a classifier was constructed only on the basis of the training set described above. Second, a final classifier was constructed on the basis of all available mass peaks and all pancreatic cancer and pancreatitis samples, fusing the corresponding training and test data sets.

Forward variable selection was applied in order to determine highly informative sets of variables ("patterns") for classification. The results of the present invention were generated using the "CART" decision tree approach (classification and regression trees; Breiman et al., 1984). Moreover, bagging of classifiers was applied to overcome typical instabilities of forward variable selection procedures, thereby increasing overall classifier performance (Breiman, 1994).

More precisely, for the training set 50 bootstrap samples were generated (sampling with replacement, maximal 3 sample redraws). For each bootstrap sample an exploratory decision tree was generated. Nodes were split using the Gini rule until all final nodes were either pure, i.e., contained only samples of one class, or until one of the following stopping rules was met: no nodes comprising less than 4 cases were split and no splits were considered resulting in a node comprising only one sample. The such obtained 50 single classifiers, one for each bootstrap sample, were combined to constitute an ensemble of classifiers predicting class membership by plurality vote.

The procedure of classifier construction was conducted twice to obtain a proof-of-principle classifier and a final classifier for pancreatic cancer detection.

Example 5. Classifier structure.

The proof-of-principle classifier employed 62 masses (variables) out of 104 determined signal clusters. Single decision trees consisted of 5 to 11 variables (6 to 12 end nodes), 6-8 variables being typical, see histogram of Figure 5. Variable importance was roughly deduced by overall improvement, i.e., for each mass we summed the improvement values achieved in the generation of all 50 decision trees of the decision tree ensemble. The masses used by the proof-of-principle classifier are listed in Table 1 (starting with most important masses having high improvement). An overview of the distribution of masses is given in Figure 6.

The final classifier for pancreatic cancer versus pancreatitis employed 71 masses out of 104 determined signal clusters. Among the 71 masses, 57 masses were already included in the proof-of-principle classifier. Single decision trees consisted of more variables than in the proof-of-principle classifier: 9-14 variables were typical, see histogram of Figure 7. Variable importance was roughly deduced by overall improvement. The masses used by the final classifier are listed in Table 2 (starting with most important masses, i.e. masses with highest improvement values). An overview of the distribution of masses of the final classifier is given in Figure 8.

The proof-of-principle classifier and the final classifier employed altogether 76 masses out of 104 determined signal clusters.

Example 6. Classification performance.

Classification performance is determined for the proof-of-principle classifier on the pancreatic cancer and pancreatitis test data. The classifier achieved 80% sensitivity and 57% specificity.

Table 1: Ranking of masses of proof-of-principle classifier by overall improvement.

mass	improvement	mass	improvement
9099	4.58	4649	0.15
7637	2.84	18304	0.15
8494	2.20	4836	0.15
8237	1.59	4875	0.14
4249	1.25	4614	0.14
11713	1.00	9163	0.14
28124	0.69	4151	0.13
2020	0.54	5497	0.13
4364	0.51	4307	0.13
3951	0.49	5119	0.11
11408	0.42	15909	0.11
18424	0.38	28009	0.10
10455	0.37	9312	0.10
11558	0.37	4725	0.09
1624	0.36	5657	0.08
4480	0.36	6458	0.07
23166	0.36	18153	0.06
11488	0.29	17932	0.06
4551	0.25	5857	0.05
22981	0.25	8596	0.05
19434	0.23	16141	0.05
8794	0.22	11241	0.05
2271	0.22	8942	0.04
15858	0.21	7013	0.04
9443	0.21	9502	0.04
8001	0.20	9652	0.03
4109	0.20	9741	0.03
17426	0.19	8717	0.03
12648	0.18	10748	0.02
9220	0.18		0.02
10233	0.15	9604	0.01

Table 2: Ranking of masses of final classifier by overall improvement.

mass	improvement	mass	improvement	mass	improvement
8494	4.160	4614	0.247	17426	0.092
9099	2.438	8794	0.244	19434	0.089
7637	1.848	22981	0.236	4109	0.088
2020	1.039	5119	0.227	10233	0.084
28124	0.973	8237	0.222	15378	0.081
1624	0.698	8001	0.204	5857	0.077
11558	0.608	8942	0.190	10455	0.076
12648	0.563	13800	0.170	9382	0.074
11713	0.559	4836	0.164	15168	0.073
10748	0.536	9220	0.163	9443	0.072
4249	0.485	4364	0.160	16141	0.069
15858	0.476	4875	0.160	18304	0.069
9163	0.441	4725	0.129	8717	0.067
3951	0.438	14206	0.122	11408	0.066
2271	0.408	6908	0.118	6458	0.060
4480	0.399	9312	0.118	5657	0.056
14829	0.362	15984	0.117	18647	0.048
4151	0.342	4969	0.115	18424	0.042
11488	0.341		0.113	4307	0.038
7013	0.337	9741	0.111	13824	0.034
4551	0.289	6866	0.108	16200	0.032
4649	0.274	23166	0.098	9652	0.017
5497	0.260	28009	0.096	16384	0.010
18153	0.258	11241	0.095		

We claim:

1. A method for the differential diagnosis of pancreatitis and/or a pancreatic cancer, *in vitro*, comprising:
   a) obtaining a test sample from a subject,
   b) contacting test sample with a biologically active surface under specific binding conditions
   c) allowing the biomolecules within the test sample to bind said biologically active surface,
   d) detecting bound biomolecules using a detection method, wherein the detection method generates a mass profile of said test sample,
   e) transforming the mass profile into a computer readable form, and
   f) comparing the mass profile of e) with a database containing mass profiles specific for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having pancreatic cancer, subjects having metastasised pancreatic cancer, or subjects having pancreatitis,

   wherein said comparison allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer and/or pancreatitis.

2. The method of claim 1, wherein the database is generated by
   a) obtaining biological samples from healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having pancreatic cancer, subjects having metastasised pancreatic cancer, and subjects having pancreatitis,
   b) contacting said biological samples with a biologically active surface under specific binding conditions,
   c) allowing the biomolecules within the biological samples to bind to said biologically active surface,
   d) detecting bound biomolecules using a detection method, wherein the detection method generates mass profiles of said biological samples,
   e) transforming the mass profiles into a computer-readable form,
   f) applying a mathematical algorithm to classify the mass profiles in e) as specific for healthy subjects, subjects having a precancerous lesion of the pancreas, subjects having pancreatic cancer, subjects having metastasised pancreatic cancer, and subjects having pancreatitis.

3. The method of claim 1, wherein the biomolecules are characterized by:

a) diluting a sample 1:5 in a denaturation buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% Ampholine, at 0° to 4°

b) further diluting said sample 1:10 with a binding buffer consisting of 0.1 M Tris-HCl, 0.02% Triton X-100, pH 8.5 at 0° to 4°

c) contacting the sample with a biologically active surface comprising positively charged quaternary ammonium groups,

d) incubating of the treated sample with said biologically active surface for 120 minutes under temperatures between 20 and 24°C at pH 8.5,

e) and analysing the bound biomolecules by gas phase ion spectrometry.

4. The method of claim 1, wherein the detection method is mass spectrometry.

5. The method of claim 4 wherein the method of mass spectrometry is selected from the group of matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), surface enhanced laser desorption ionisation/time of flight (SELDI-TOF), liquid chromatography, MS-MS, or ESI-MS.

6. The method of claims 1, wherein the biologically active surface comprises an adsorbent selected from the group of quaternary ammonium groups, carboxylate groups, groups with alkyl or aryl chains, groups such as nitriloacetic acid that immobilize metal ions, or proteins, antibodies, or nucleic acids.

7. The method of claim 1, wherein the mass profiles comprise a panel of one or more differentially expressed biomolecules.

8. The method of claim 7, wherein, wherein the biomolecules are selected from a group having the apparent molecular mass of 1624 Da ± 8 Da, 2020 Da ± 10 Da, 2271 Da ± 11 Da, 3951 Da ± 20 Da, 4108 Da ± 20 Da, 4151 Da ± 21 Da, 4249 Da ± 21 Da, 4307 Da ± 22 Da, 4364 Da ± 22 Da, 4480 Da ± 22 Da, 4551 Da ± 23 Da, 4614 Da ± 23 Da, 4649 Da ± 23 Da, 4725 Da ± 24 Da, 4836 Da ± 24 Da, 4875 Da ± 24 Da, 4969 Da ± 25 Da, 5119 Da ± 26 Da, 5497 Da ± 27 Da, 5657 Da ± 28 Da, 5857 Da ± 29 Da, 6458 Da ± 32 Da, 6866 Da ± 34 Da, 6908 Da ± 35 Da, 7013 Da ± 35 Da, 7637 Da ± 38 Da, 8001 Da ± 40 Da, 8237 Da ± 41 Da, 8494 Da ± 42 Da, 8596 Da ± 43 Da, 8717 Da ± 44 Da, 8794 Da ± 44 Da, 8942 Da ± 45 Da, 9099 Da ± 45 Da, 9163 Da ± 46 Da, 9220 Da ± 46 Da, 9312 Da ± 47 Da, 9382 Da ± 47 Da, 9443 Da ± 47 Da, 9502 Da ± 48 Da, 9604 Da ± 48 Da, 9652 Da ± 48 Da, 9741 Da ± 49 Da, 10233 Da ± 51 Da, 10455 Da ± 52 Da, 10748 Da ± 54 Da, 11241 Da ± 56 Da, 11408 Da ± 57 Da, 11488 Da ± 57 Da, 11558

Da ± 58 Da, 11713 Da ± 59 Da, 12648 Da ± 63 Da, 13800 Da ± 69 Da, 13824 Da ± 69 Da, 14206 Da ± 71 Da, 14829 Da ± 74 Da, 15168 Da ± 26 Da, 15378 Da ± 77 Da, 15858 Da ± 79 Da, 15909 Da ± 78 Da, 15984 Da ± 80 Da, 16141 Da ± 81 Da, 16200 Da ± 81 Da, 16384 Da ± 82 Da, 16986 Da ± 85 Da, 17426 Da ± 87 Da, 17932 Da ± 90 Da, 18153 Da ± 91 Da, 18304 Da ± 92 Da, 18424 Da ± 92 Da, 18647 Da ± 93 Da, 19434 Da ± 97 Da, 22981 Da ± 115 Da, 23166 Da ± 116 Da, 28009 Da ± 140 Da, or 28124 Da ± 141 Da.

9. A method for the identification of differentially expressed biomolecules wherein the biomolecules of any of claims 1-8 are proteins, comprising:
   a) chromatography and fractionation,
   b) analysis of fractions for the presence of said differentially expressed proteins and/or fragments thereof, using a biologically active surface,
   c) further analysis using mass spectrometry to obtain amino acid sequences encoding said proteins and/or fragments thereof, and
   d) searching amino acid sequence databases of known proteins to identify said differentially expressed proteins by amino acid sequence comparison.

10. The method of claim 9, wherein the method of chromatography is selected from high performance liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC).

11. The method of claim 9, wherein the mass spectrometry used is selected from the group of matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), surface enhanced laser desorption ionisation/time of flight (SELDI-TOF), liquid chromatography, MS-MS, or ESI-MS.

12. A method for the differential diagnosis of pancreatitis and/or pancreatic cancer, *in vitro*, comprising detection of one or more differentially expressed biomolecules wherein the biomolecules are polypeptides, comprising:
    a) obtaining a test sample from a subject,
    b) contacting said sample with a binding molecule specific for a differentially expressed polypeptide identified in claims 9-11,
    c) detecting the presence or absence of said polypeptide(s),

    wherein the presence or absence of said polypeptide(s) allows for the differential diagnosis of a subject as healthy, having a precancerous lesion of the pancreas, having a pancreatic cancer, having a metastasised pancreatic cancer and/or pancreatitis.

13. The method or kit of any one of claims 1-12, wherein pancreatitis is the chronic form of the disease.

14. The method of any one of claims 1-12, wherein the test sample is a blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract sample.

15. The method of any one of claims 1-12, wherein the biological sample is a blood, blood serum, plasma, nipple aspirate, urine, semen, seminal fluid, seminal plasma, prostatic fluid, excreta, tears, saliva, sweat, biopsy, ascites, cerebrospinal fluid, milk, lymph, or tissue extract sample.

16. The method of any one of claims 1-12, wherein the subject is of mammalian origin.

[redacted]

18. A kit for the diagnosis of pancreatitis or a pancreatic cancer within a subject using the method of claim 1-11 and 13-17 comprising a denaturation solution, a binding solution, a washing solution, a biologically active surface comprising an adsorbent, and instructions to use the kit.

[redacted] A kit for the diagnosis of pancreatitis or a pancreatic cancer within a subject using the method of claim 12-17 comprising a solution, binding molecule, detection substrate, and instructions to use the kit.

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FIGURE 1

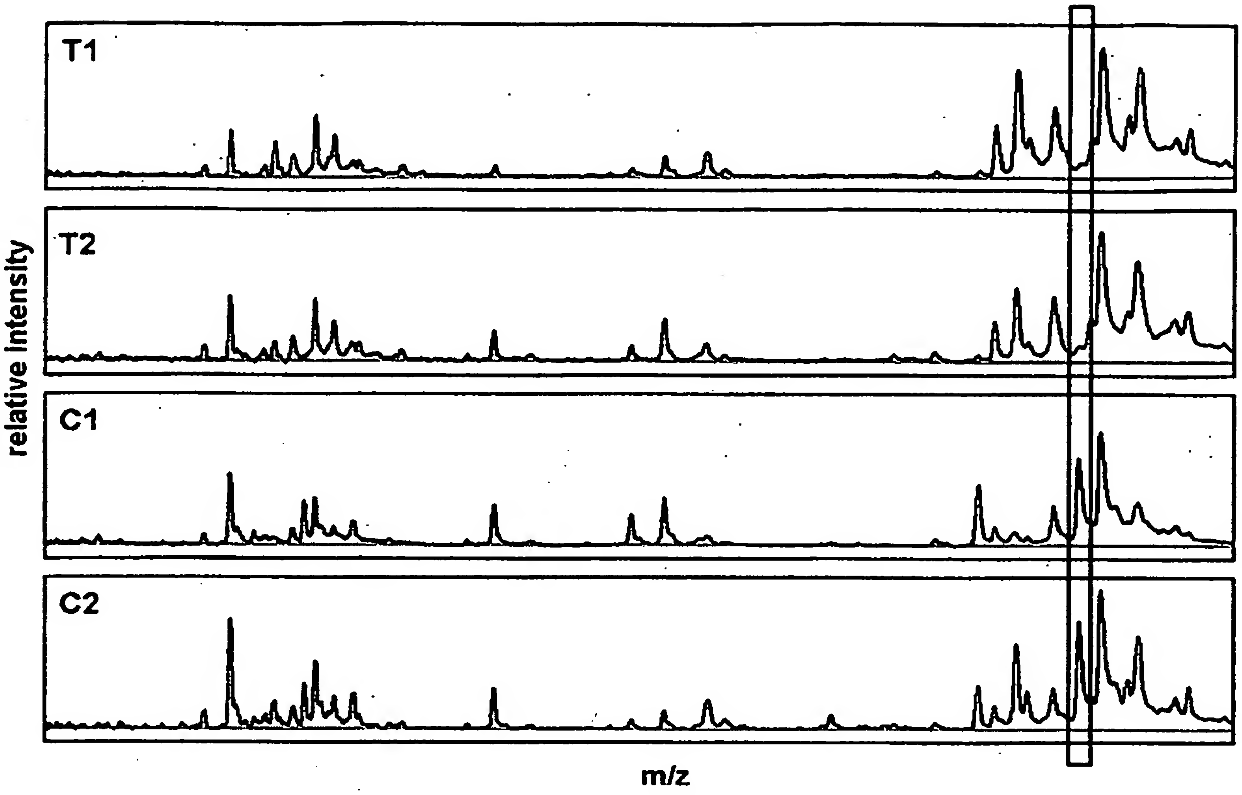

FIGURE 2A

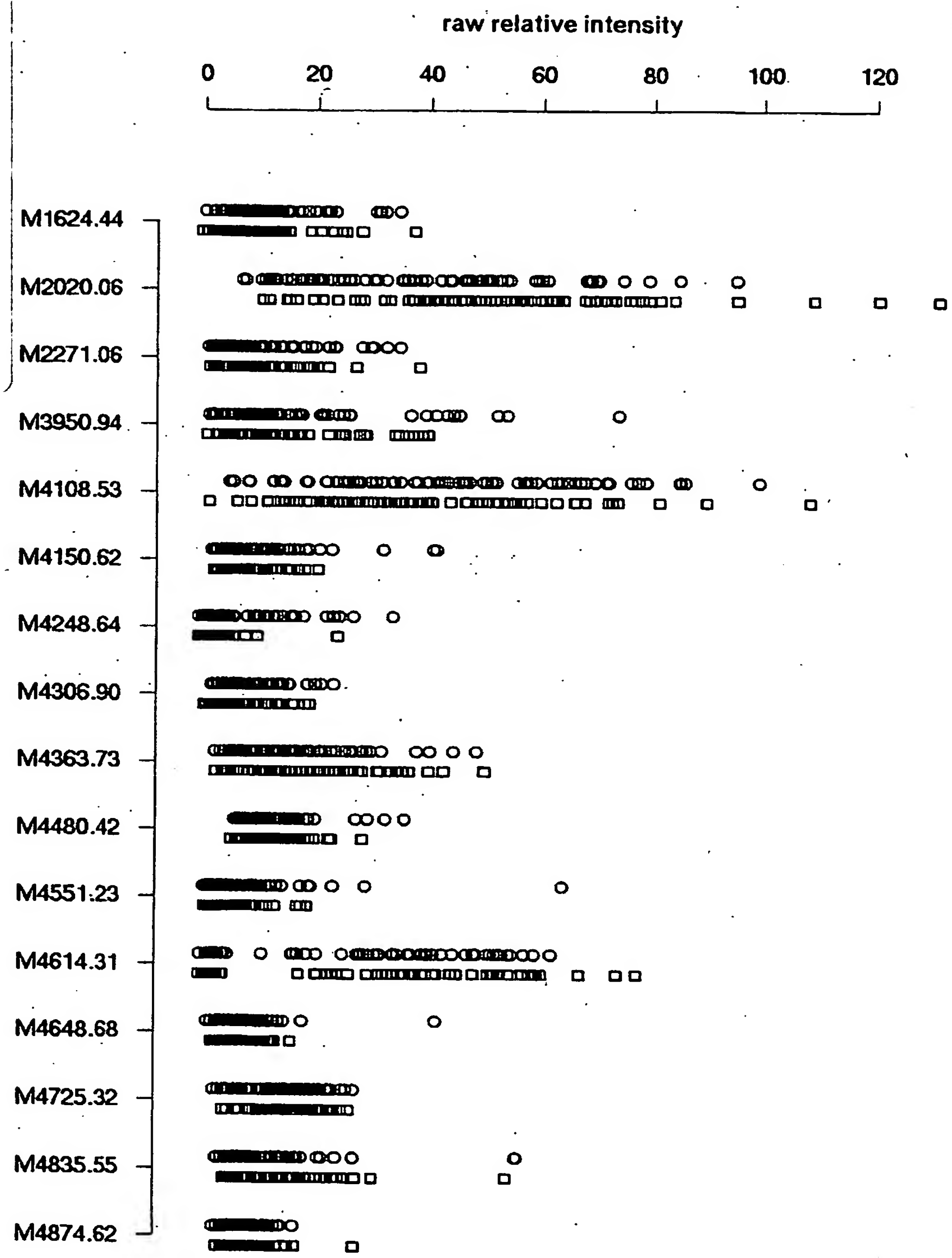
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FIGURE 2B

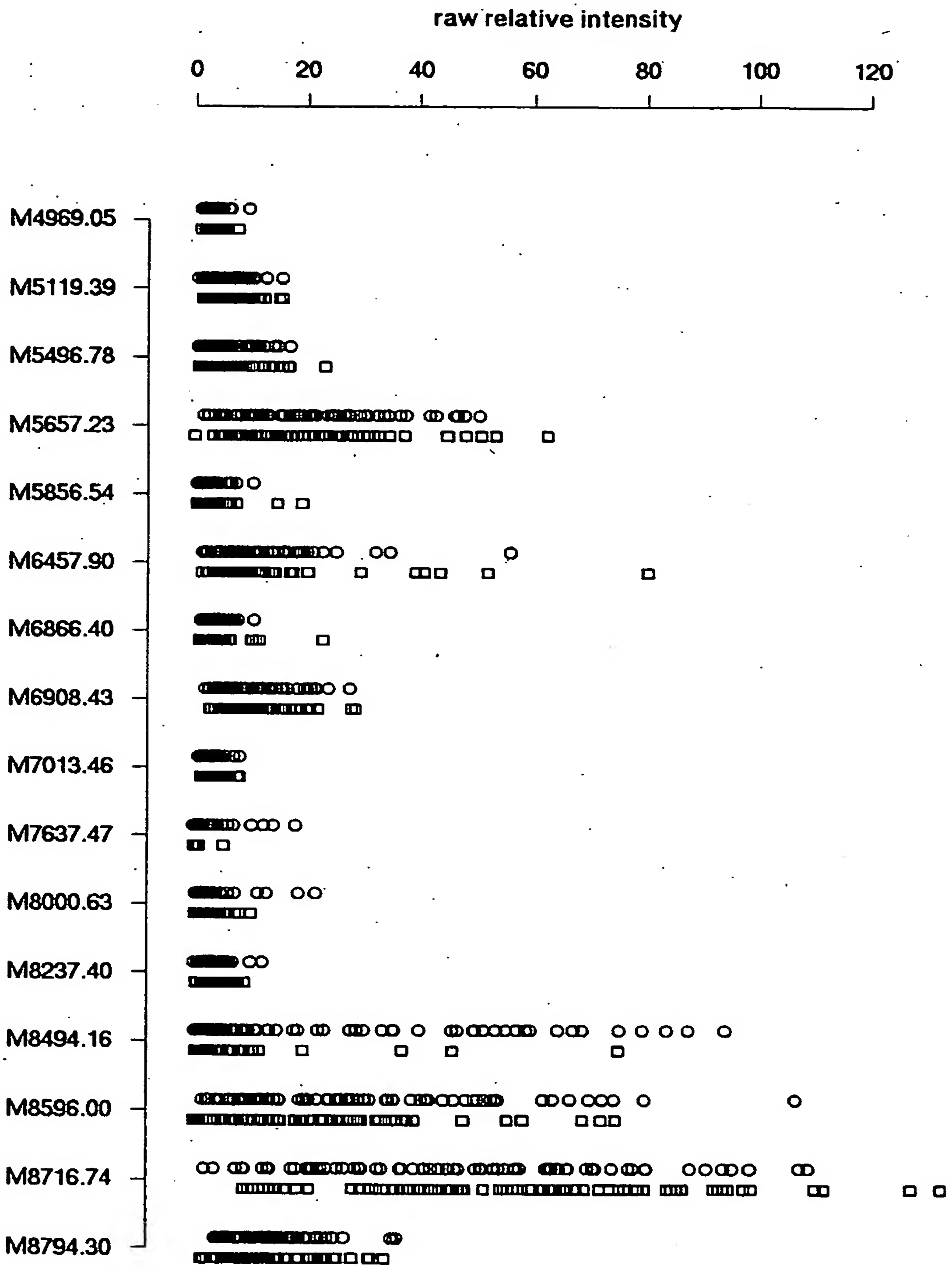

FIGURE 2C

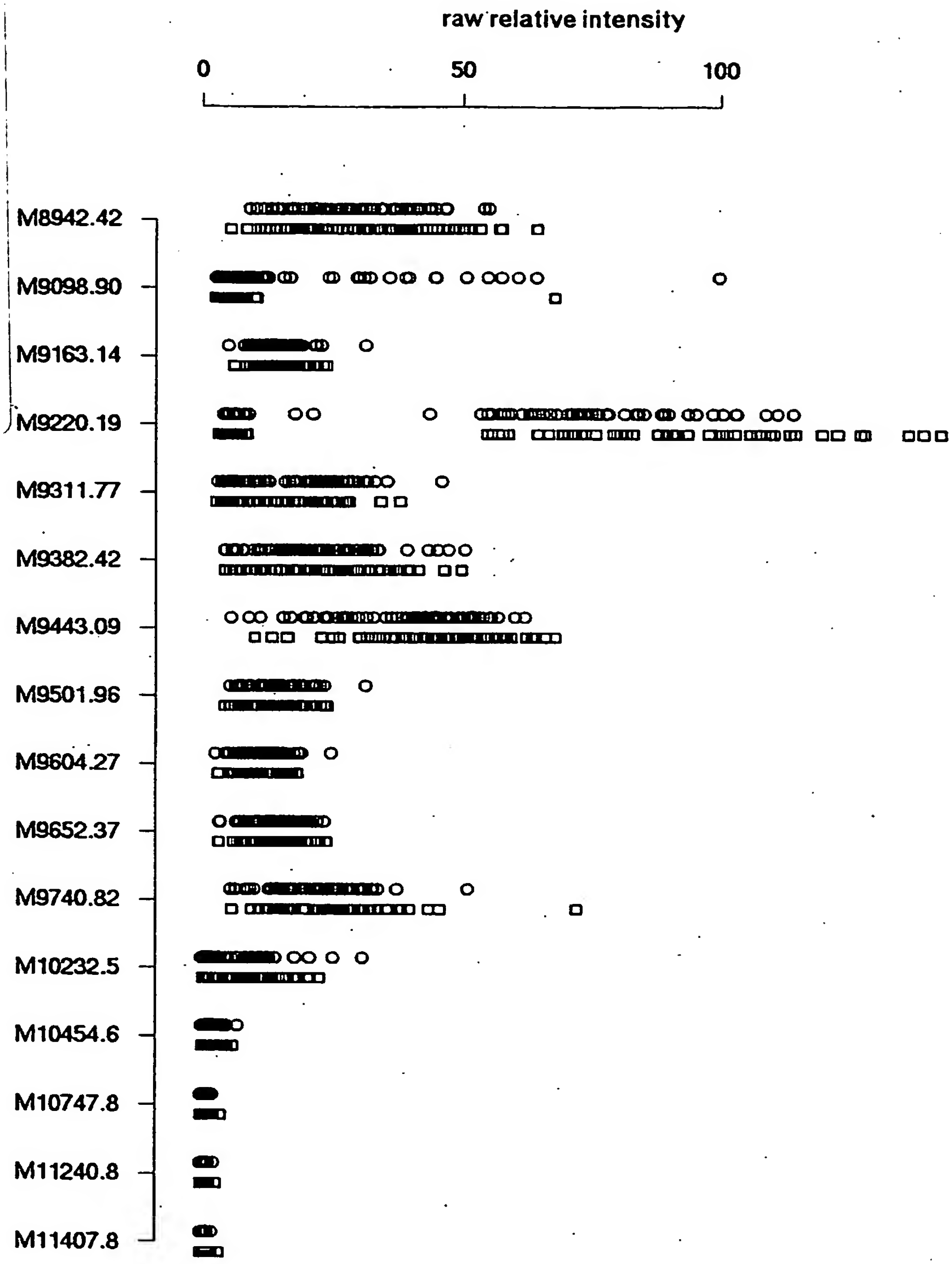
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FIGURE 2D

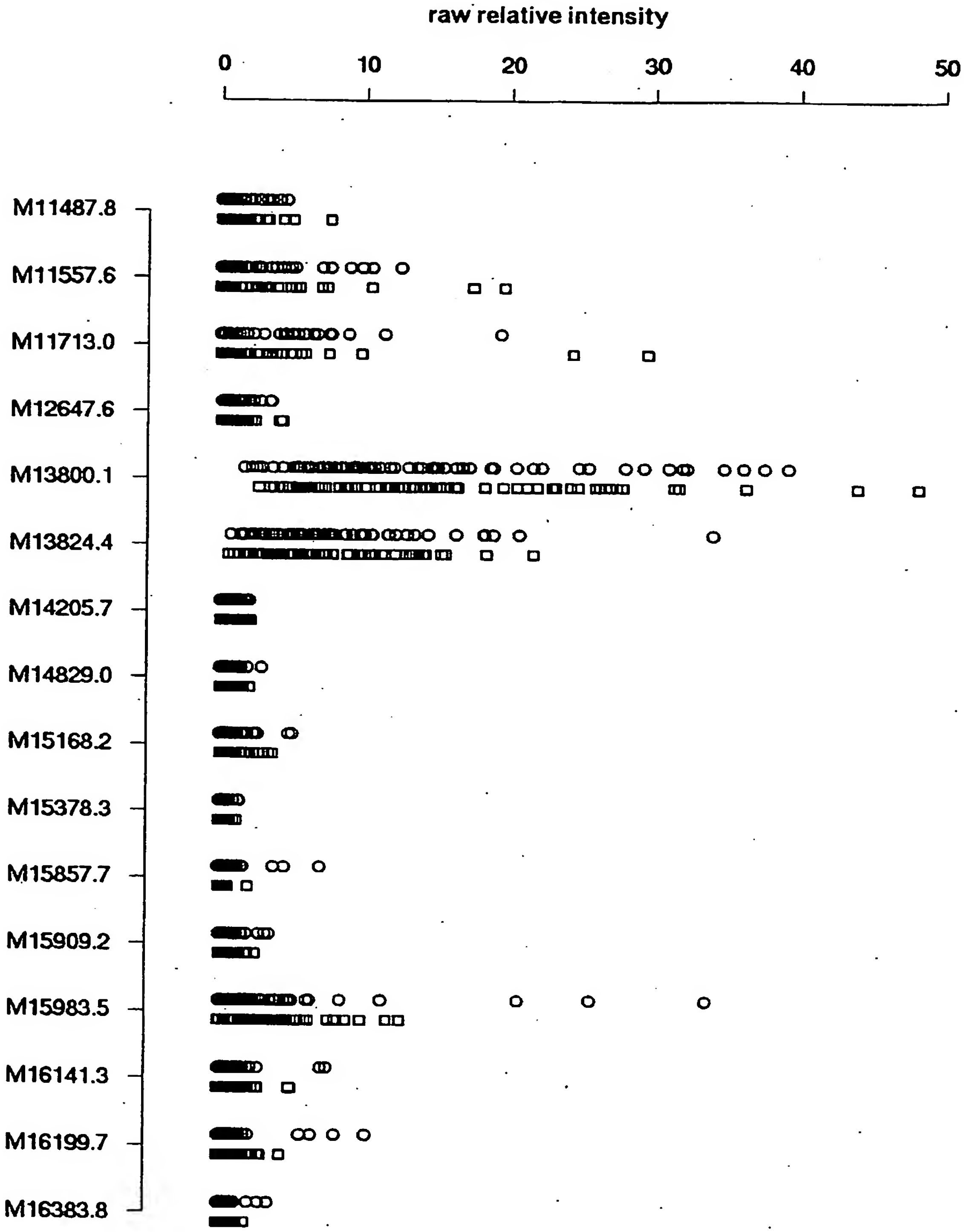

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FIGURE 2E

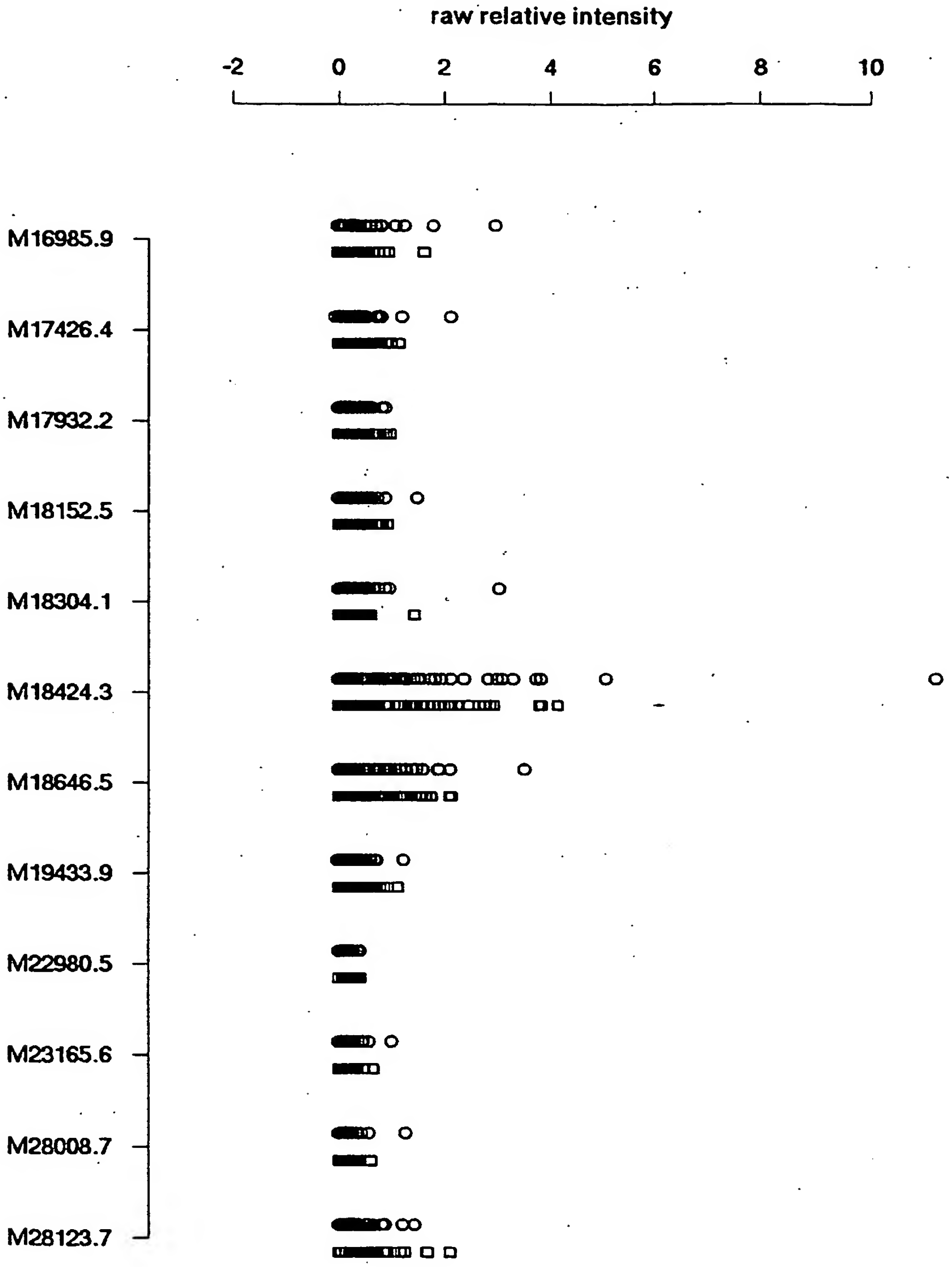

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FIGURE 3A

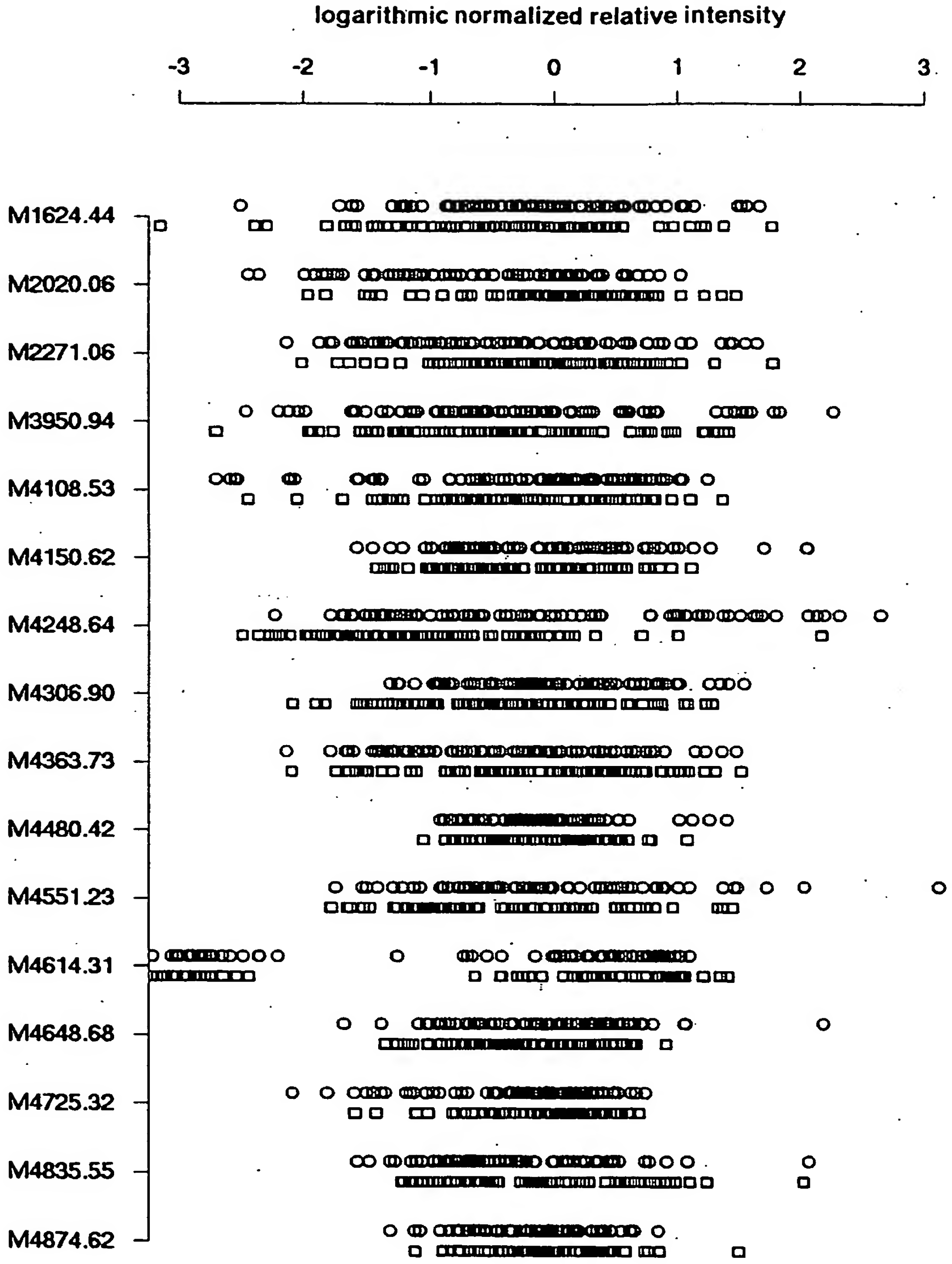

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FIGURE 3B

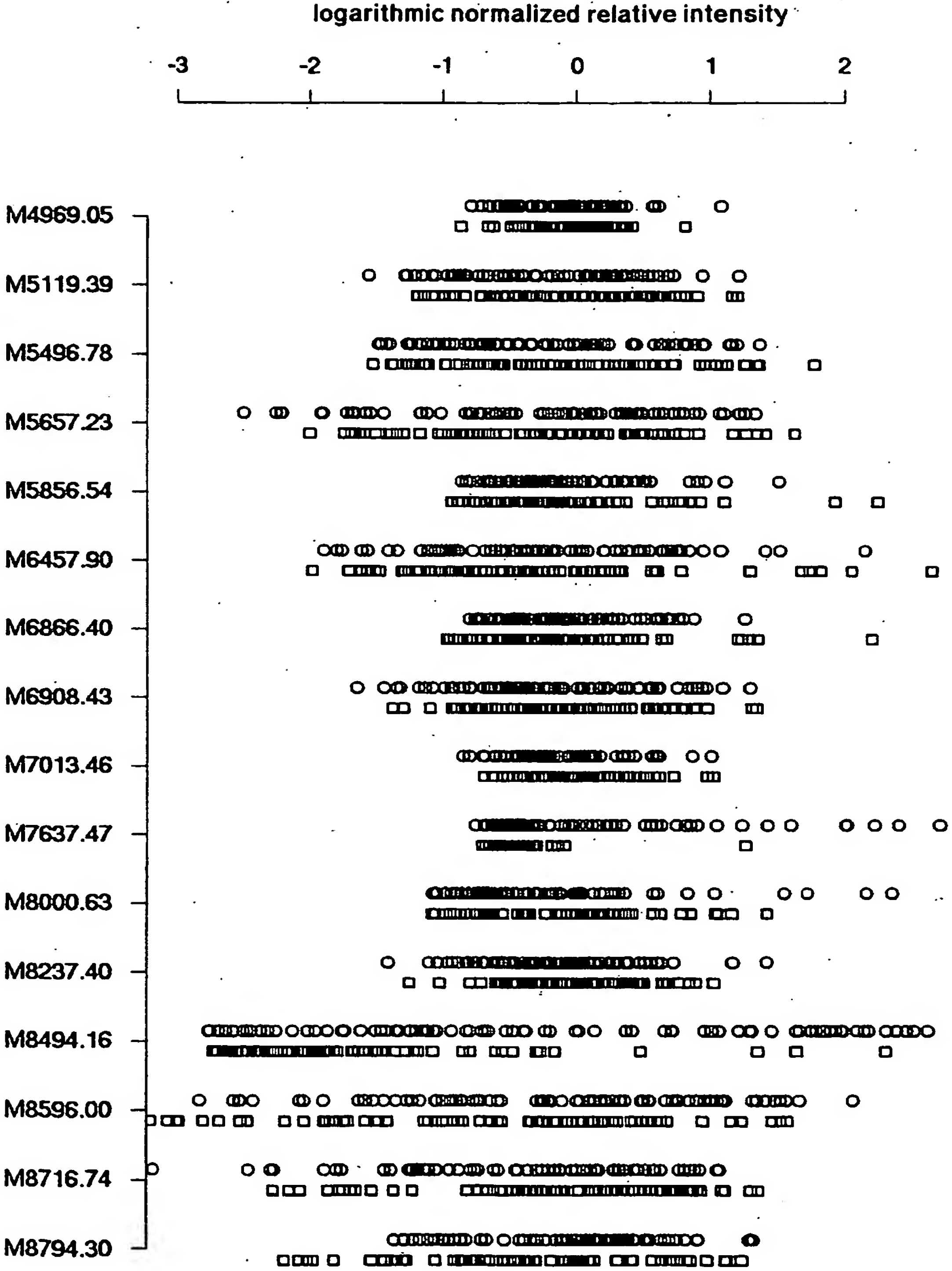

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FIGURE 3C

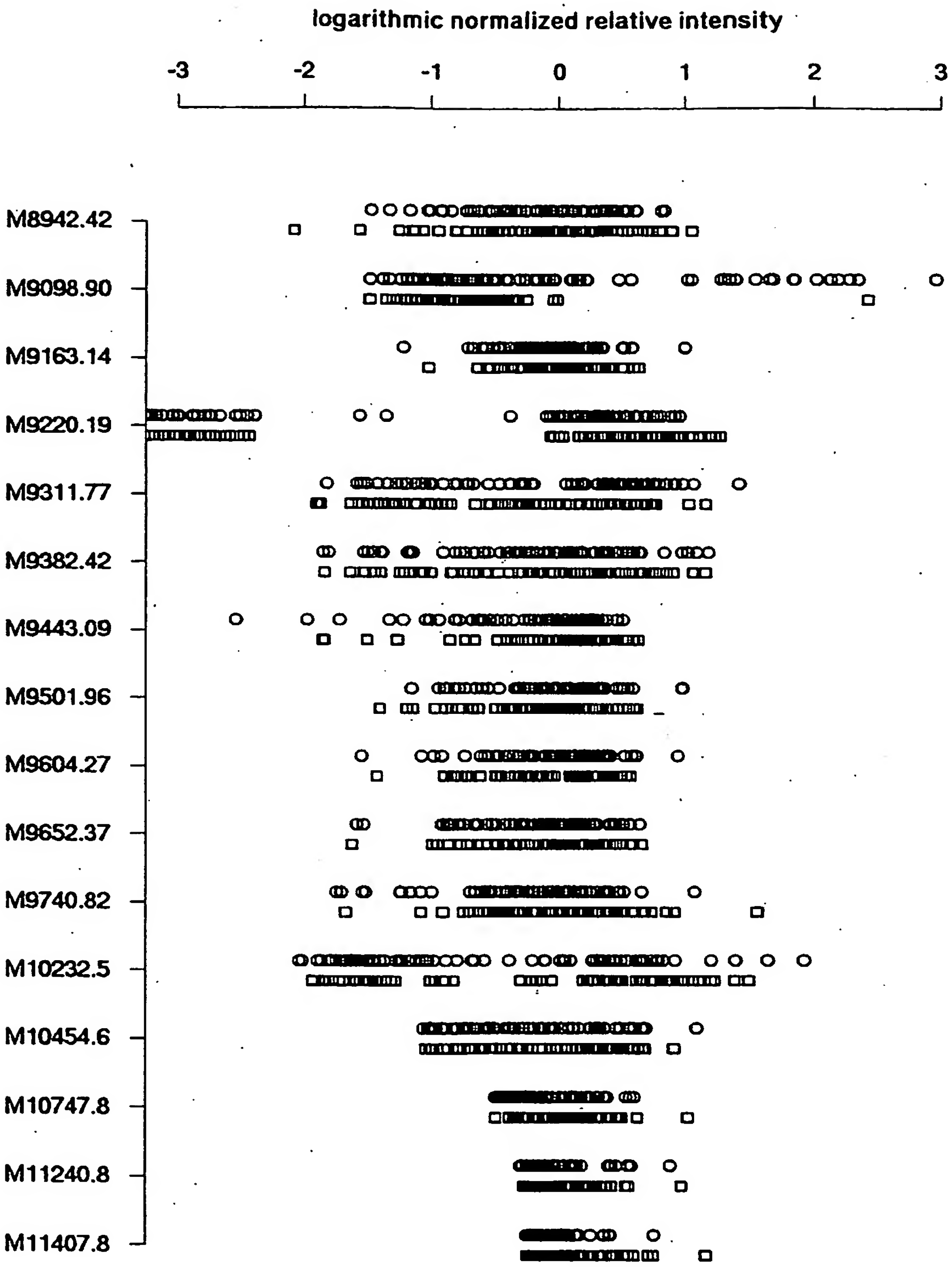

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FIGURE 3D

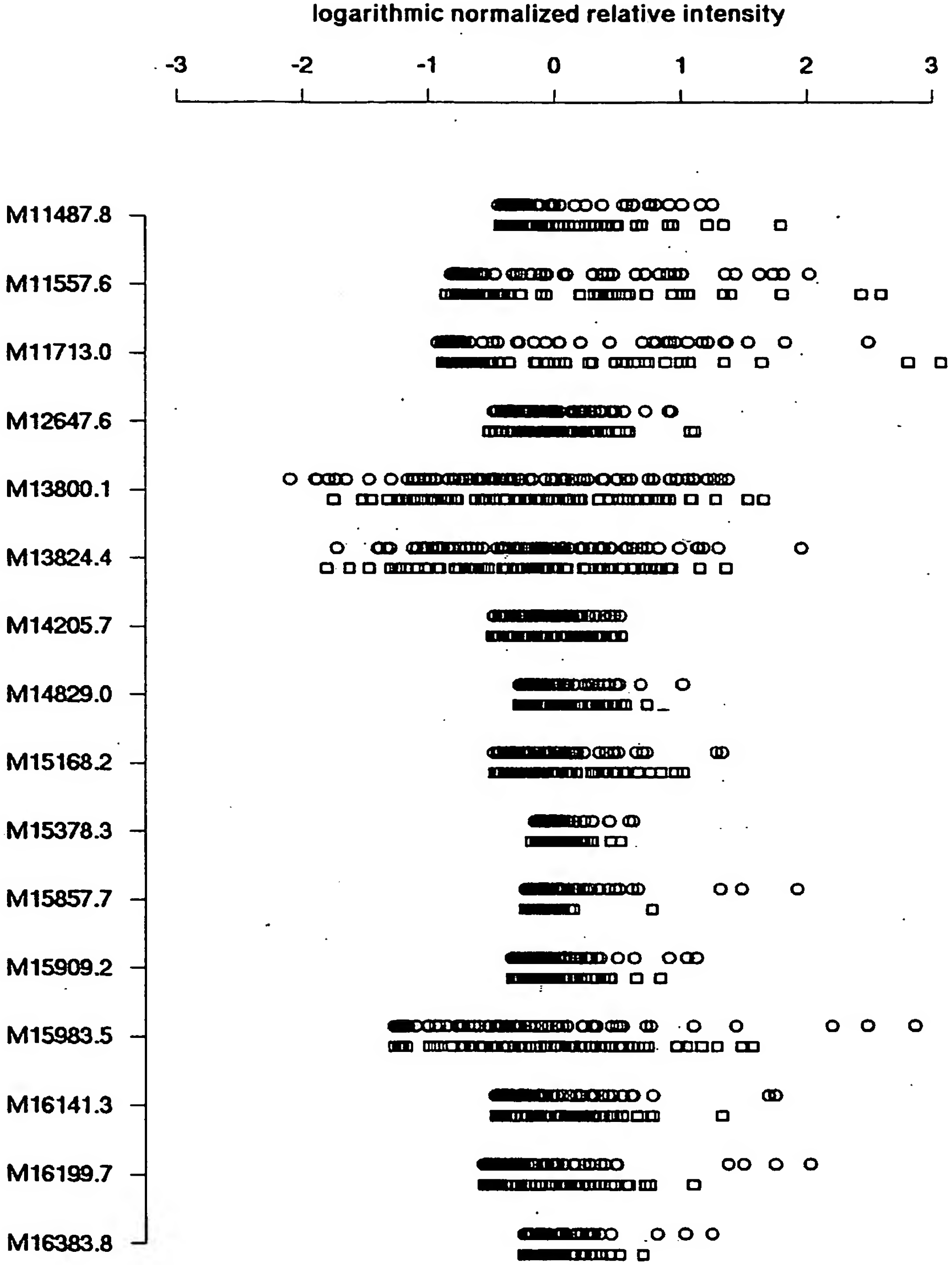

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FIGURE 3E

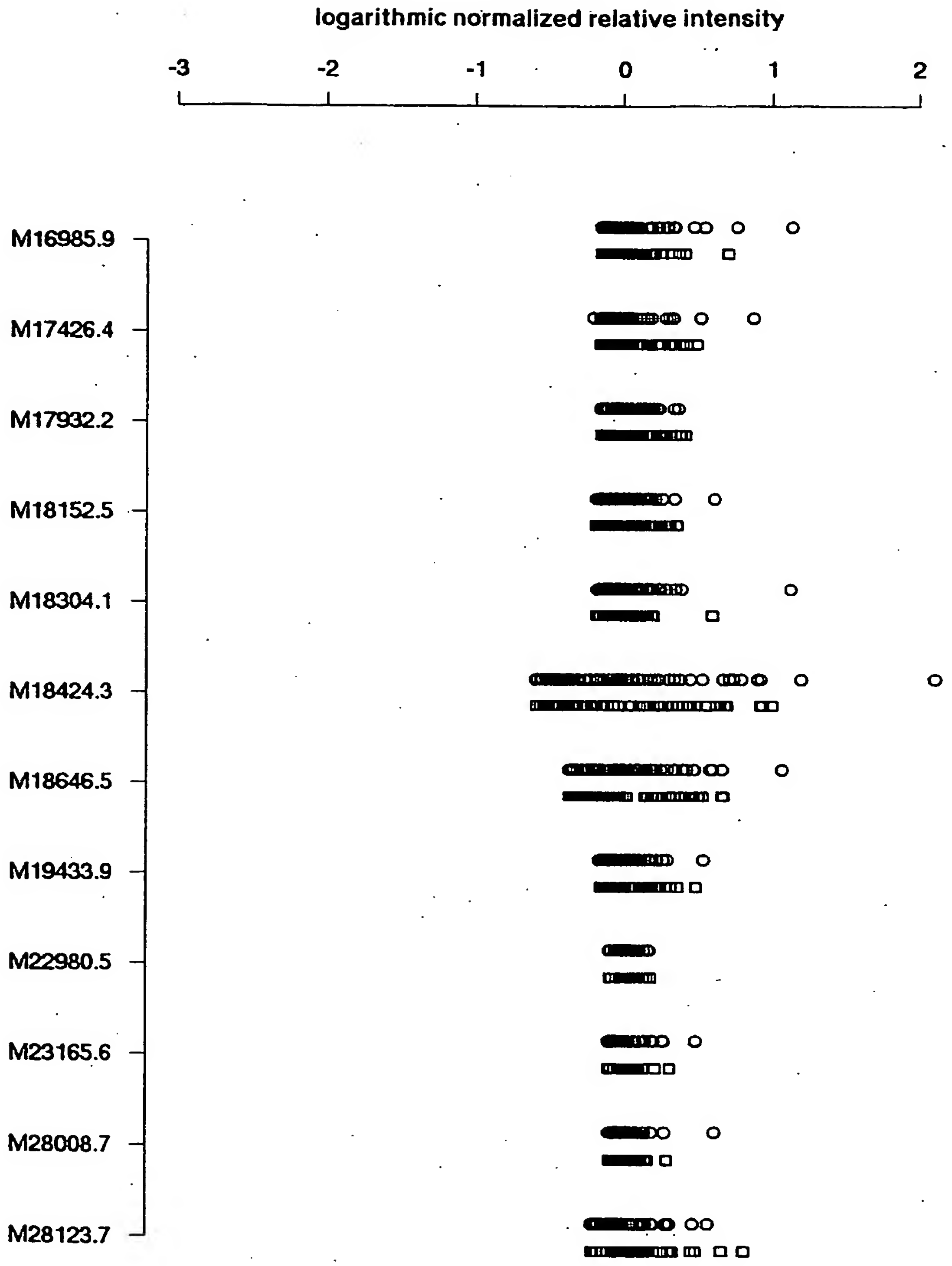

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FIGURE 4A

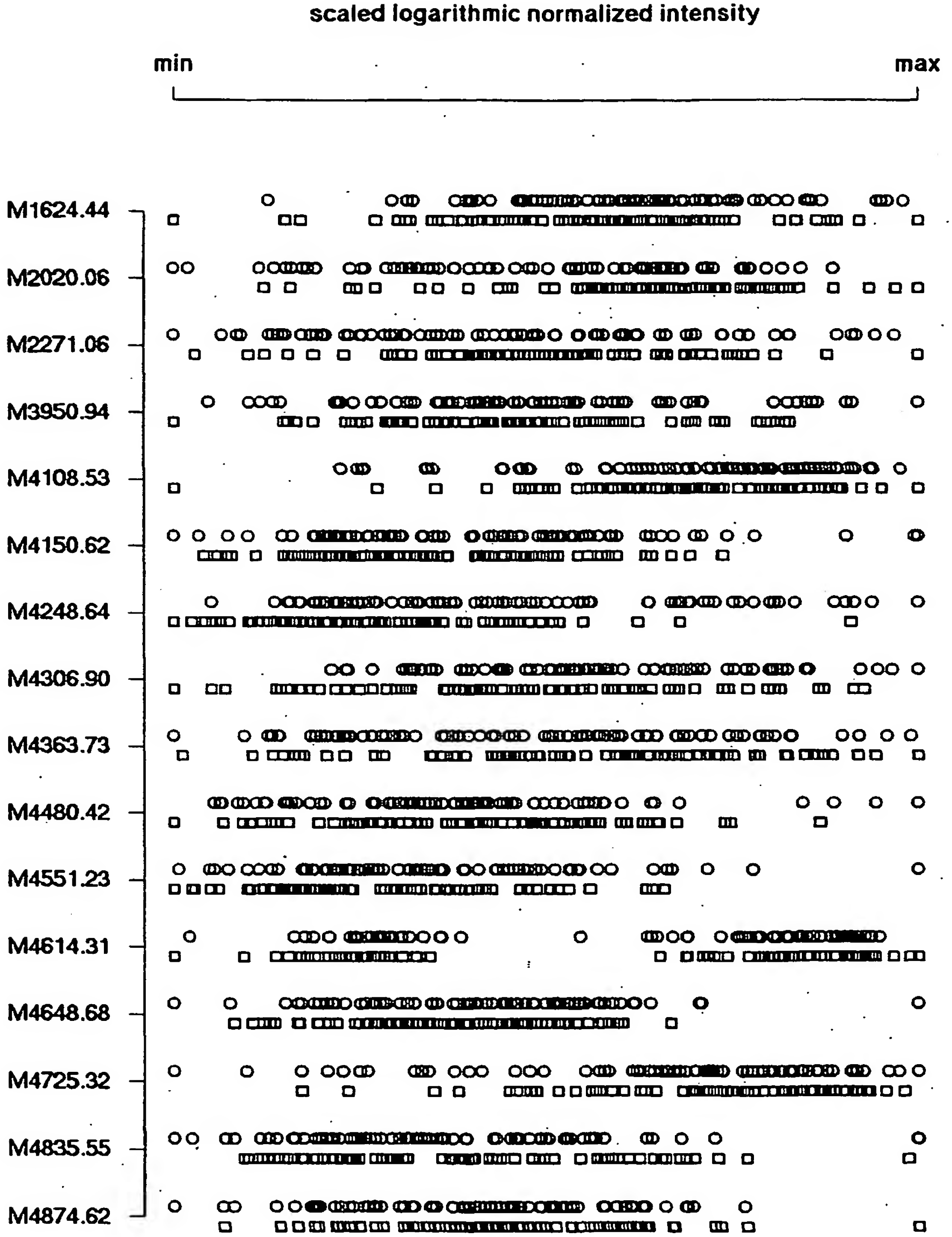

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FIGURE 4B

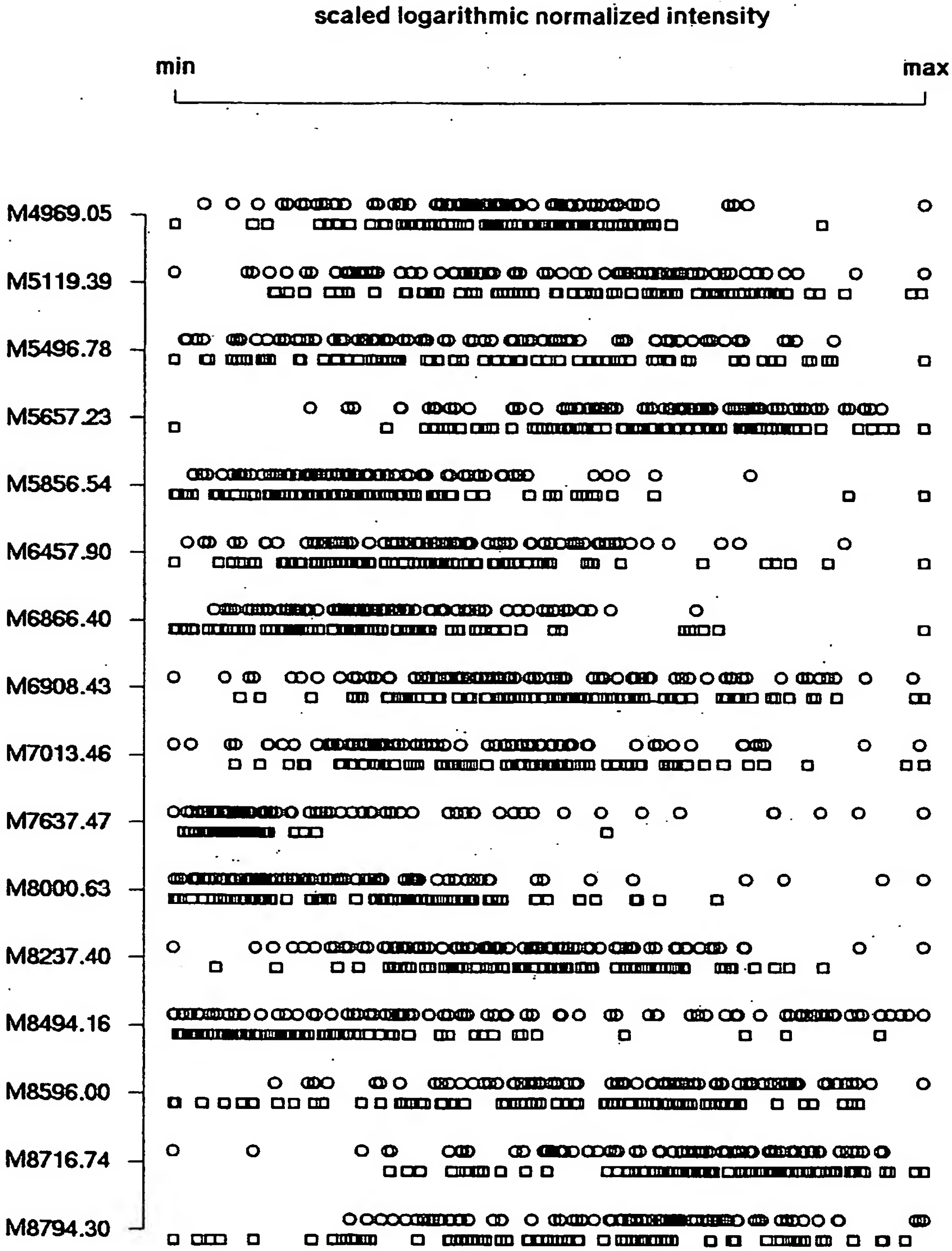

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FIGURE 4C

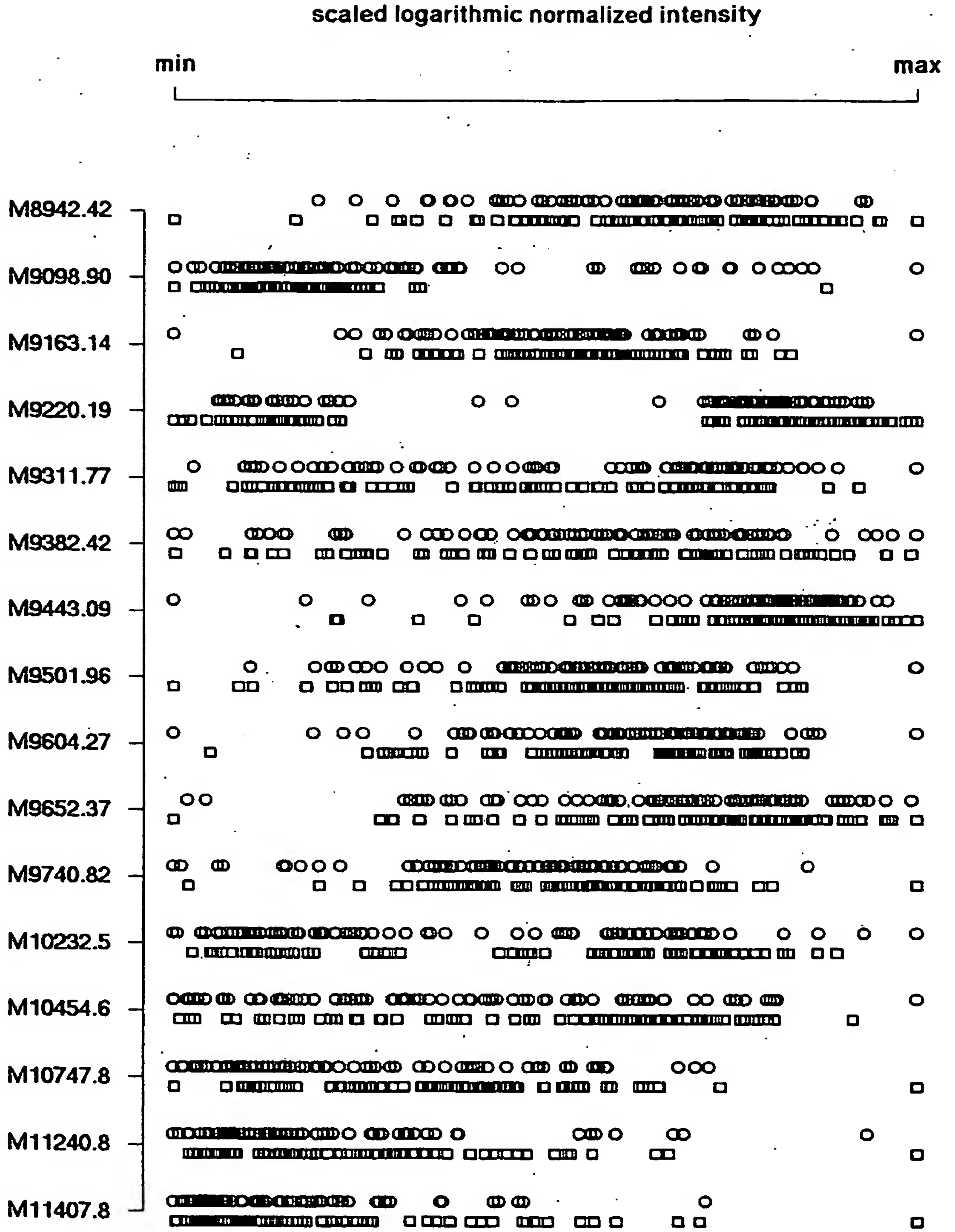

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FIGURE 4D

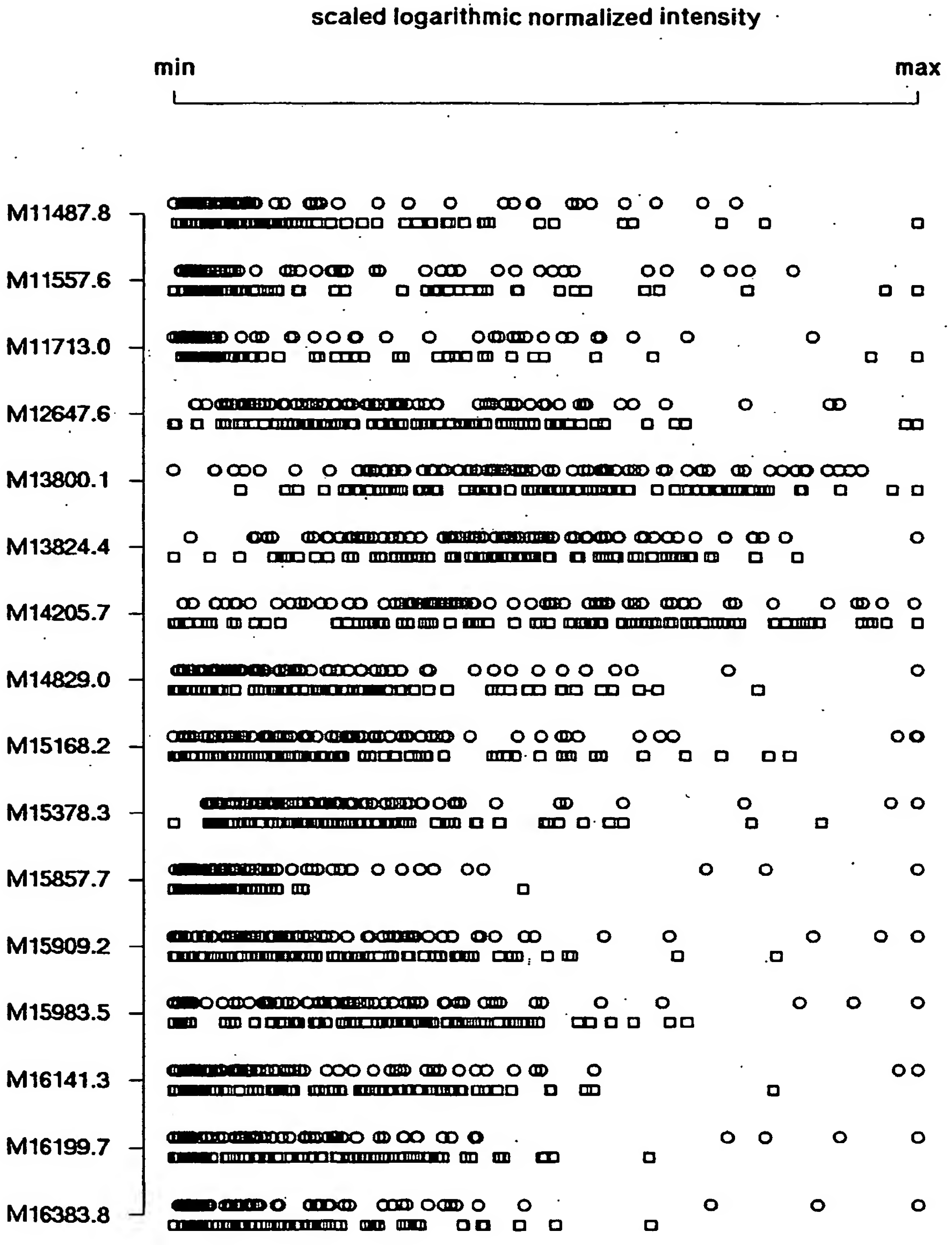

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FIGURE 4E

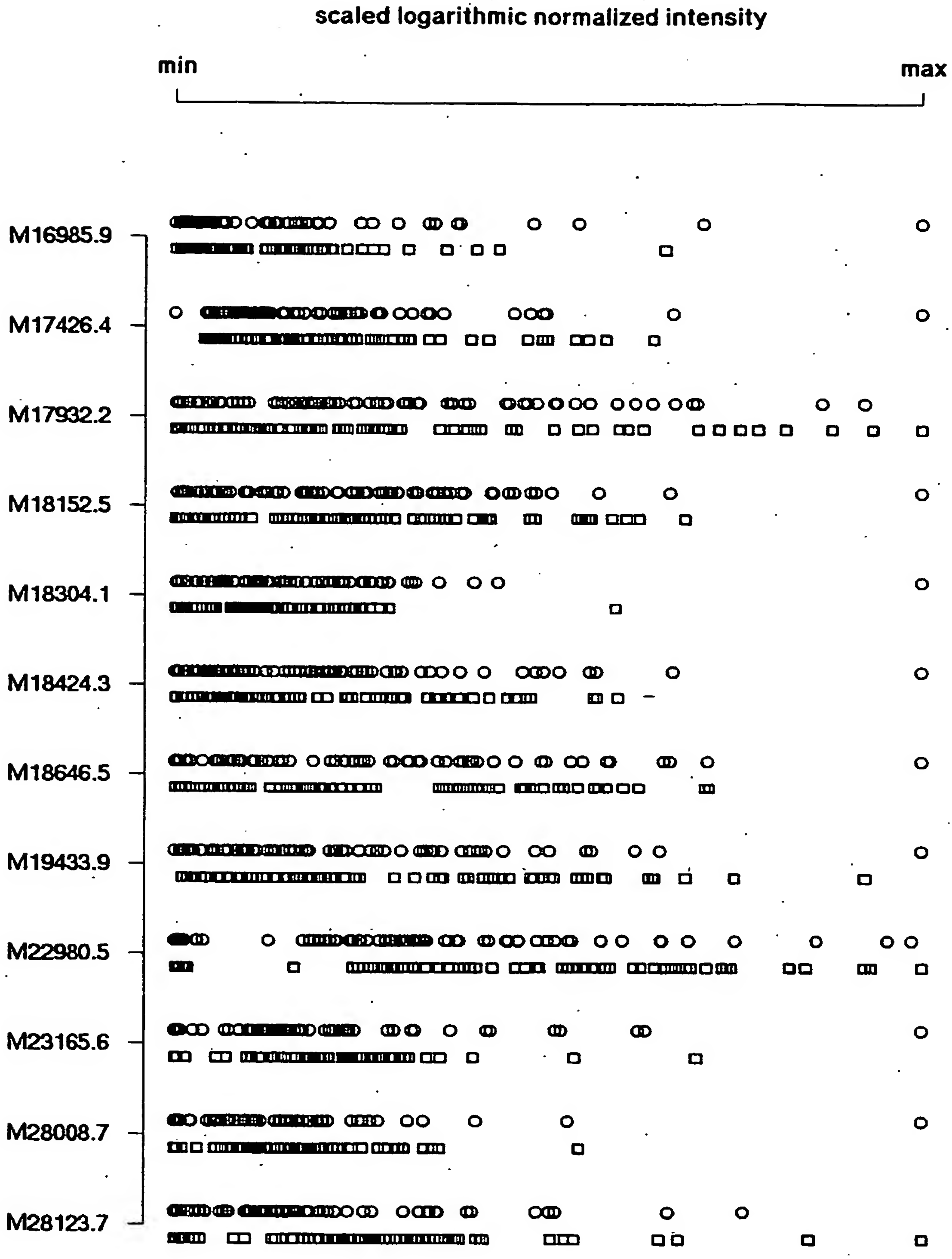

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FIGURE 5

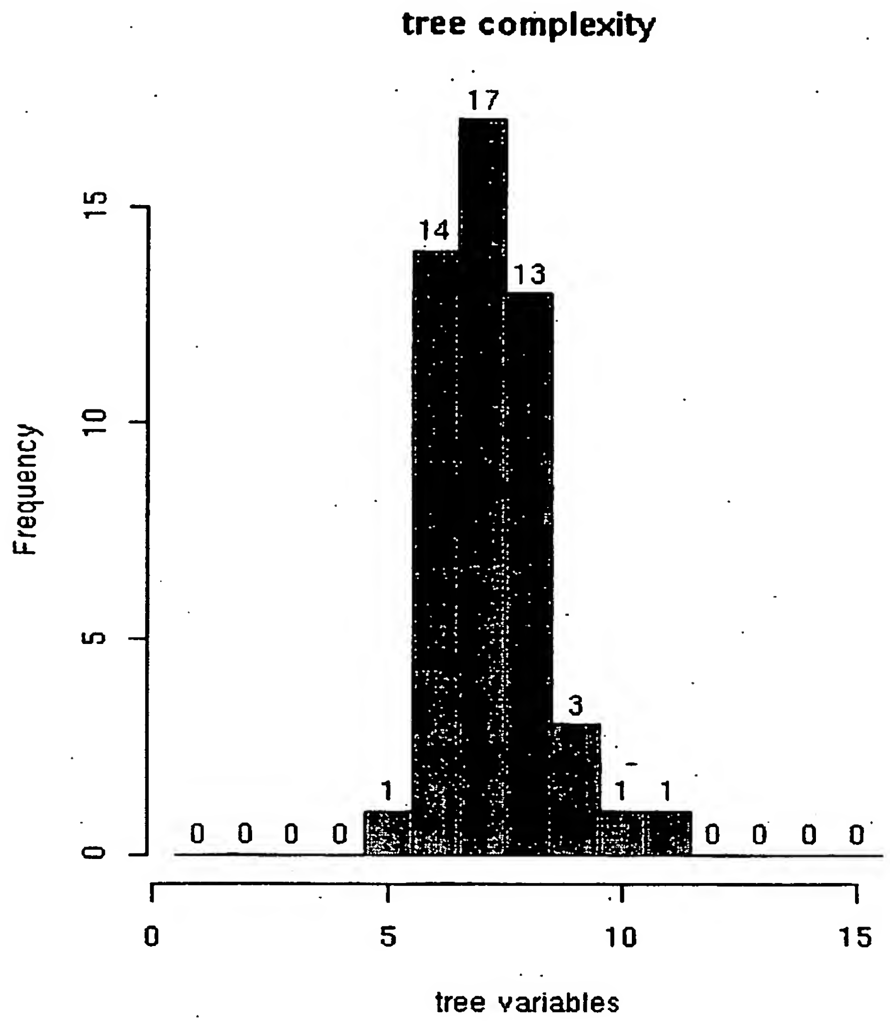

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FIGURE 6

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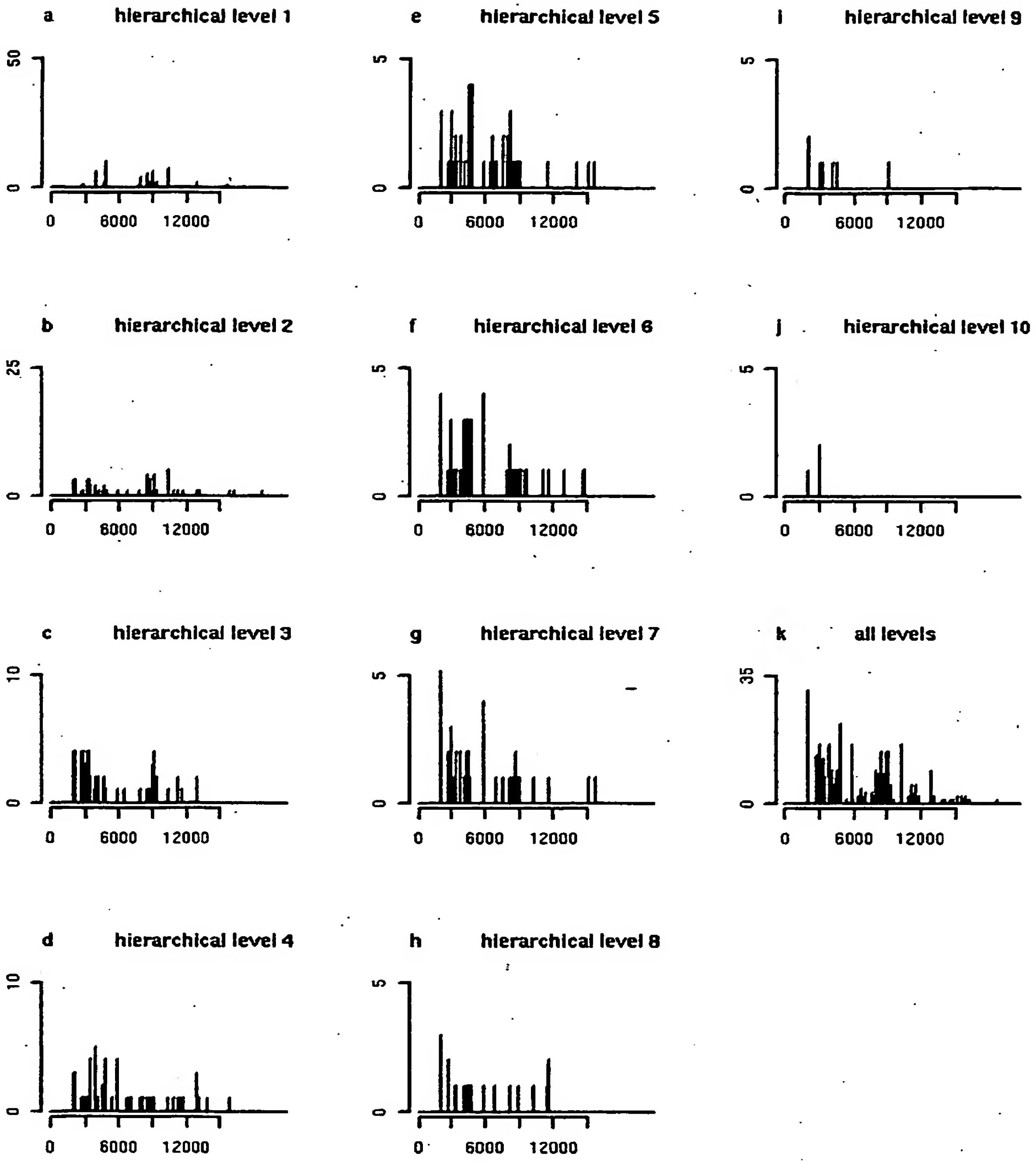

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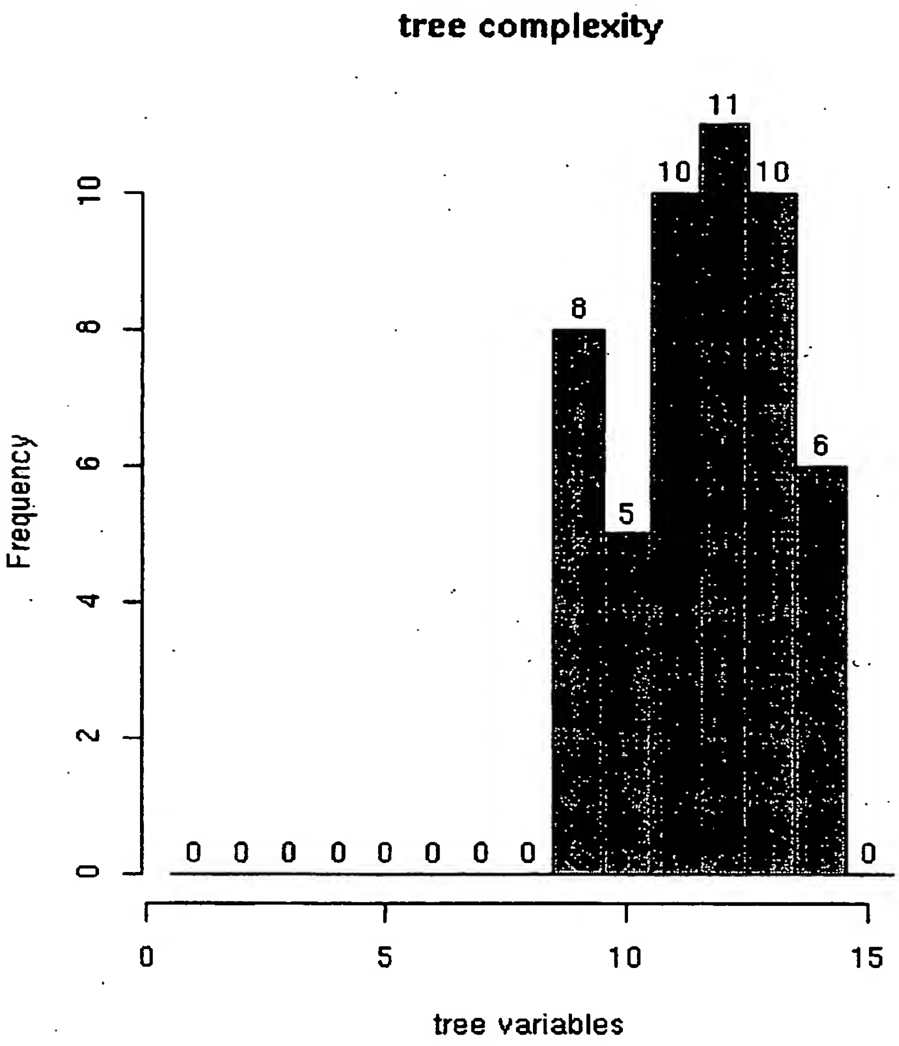
FIGURE 7



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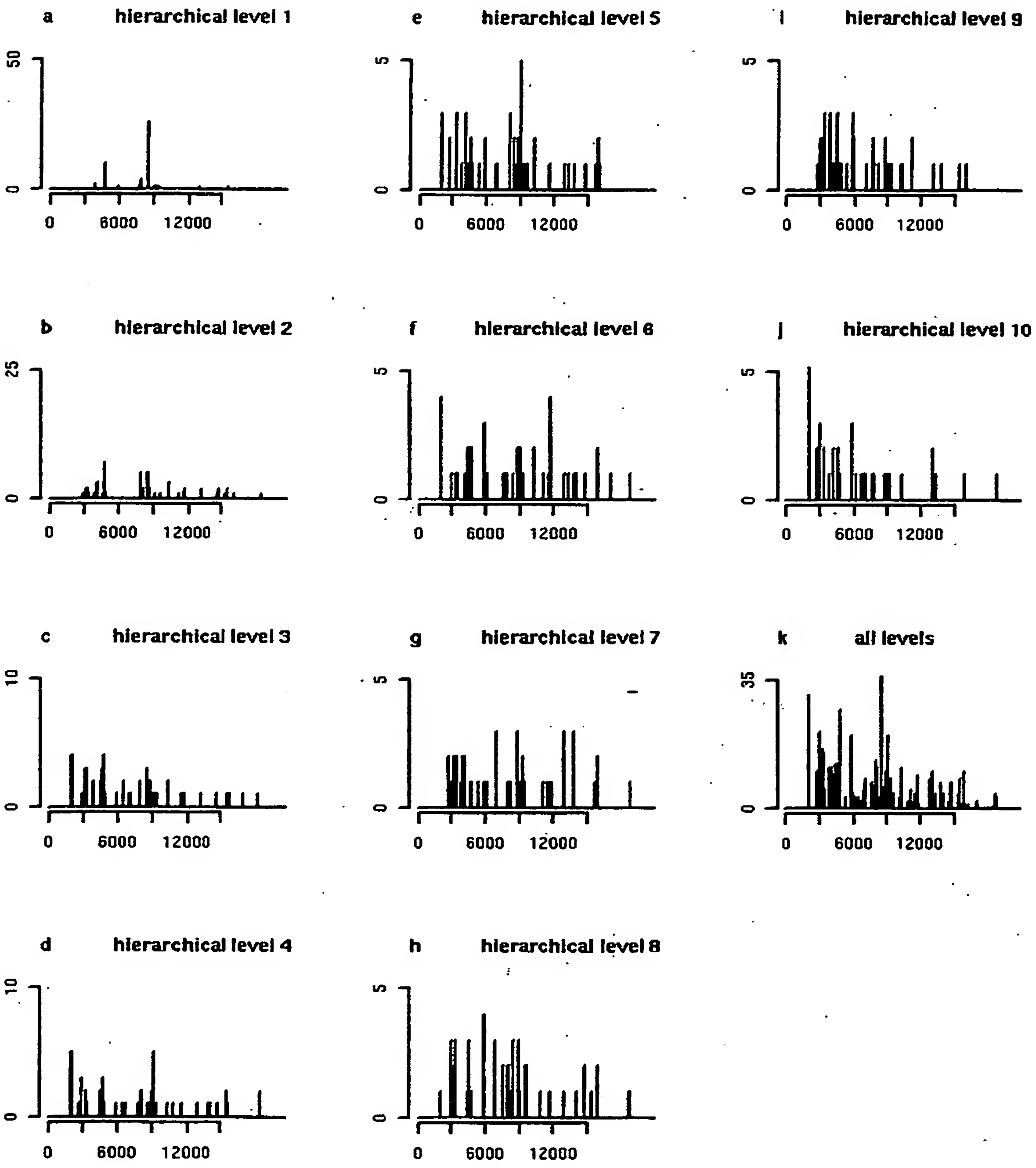FIGURE 8